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THE EFFECT OF MUSCULAR EXERCISE ON THE SERUM CHOLINESTERASE LEVEL IN NORMAL ADULTS AND IN PATIENTS WITH MYASTHENIA GRAVIS

By H. B. STONER AND A. WILSON, *From the Department of Pharmacology and Therapeutics, University of Sheffield and the Sheffield Royal Infirmary*

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METHODS

Samples of blood were taken from the antecubital vein of the left forearm before and after a period of four minutes of occlusion of the circulation by a pressure of 200 mm. of mercury, with and without exercise. The exercise consisted in flexion and extension of the fingers, pronation and supination of the wrist and flexion and extension of the wrist, performed in rotation.

Observations were made on eleven patients with myasthenia gravis. Two of these patients (Cases 16, 17) had not at any time received any prostigmin therapy. Nine cases (Nos. 7–15) were under treatment with prostigmin, and blood was withdrawn from these patients some time after they were due to receive their next dose of prostigmin. The times at which the last dose of prostigmin had been given is stated in column 1, Table 2. It should be pointed out that in all cases the investigations were carried out when the patients exhibited the signs and symptoms typical of myasthenia gravis. The serum cholinesterase levels were determined by the manometric method using a Warburg manometer. The method depends on the evolution of carbon dioxide from a bicarbonate buffer solution by the acetic acid from the substrate, acetylcholine. The experimental details are as follows:

2·5 ml. of salt solution, 1·25 ml. of 0·154 M sodium bicarbonate, and 3·65 ml.

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of distilled water were saturated with 5% carbon dioxide in nitrogen and 0·1 ml. of blood serum was added. The salt solution consisted of:

NaCl	18·0 gm.
MgSO$_4$ (0·154 M/1)	26·0 ml.
CaCl$_2$ (0·154 M/1)	77·0 ml.
KCl (0·154 M/1)	128·0 ml.
Distilled water to	1000 ml.

3 ml. (equiv. 40 μl. of serum) of this mixture were placed in the centre of the reaction flask. In the side bulb of the flask was placed 0·33 ml. of M/5 acetylcholine chloride in distilled water. The solution of acetylcholine chloride was prepared freshly each day from the same sample of acetylcholine chloride (B.D.H.) which was stored in a desiccator over calcium chloride. The apparatus was gassed with 5% carbon dioxide in nitrogen and shaken in the water bath at 37° C. until equilibrium was attained, that is in about 10 min. The taps were then closed and the acetylcholine solution was added to the main flask and shaken at about 106 oscillations per min. Readings of the manometer were taken at 5 min. intervals for the first 15 min. and thereafter at 15 min. intervals for 1 hr. The readings were plotted against time and interpolated to give a one hour value. The activity of the serum, Q_{CO_2}, was then calculated from the formula: pressure change per hr. in mm. multiplied by the vessel constant divided by μl. of serum. All estimations were done in duplicate. The unit of cholinesterase activity (Q_{CO_2}) thus represents μl. CO_2 per μl. of serum per hour.

RESULTS

The results of these estimations are tabulated in Tables 1, 2 and 3.

TABLE 1. Serum cholinesterase activity of normal adults: (a) before and after occlusion at rest; (b) before and after occlusion with exercise

	(a)			
Case no.	At rest, before occlusion (units)	At rest, after occlusion (units)	Difference	% difference in Q (CO_2)
1	5·97	6·17	+0·2	+ 3·3
2	4·63	4·95	+0·32	+ 6·9
3	5·86	6·27	+0·41	+ 7·0
4	3·57	3·79	+0·22	+ 6·2
5	5·20	5·16	−0·04	− 0·8
6	3·97	3·10	−0·87	−21·8
	(b)			
	At rest, before occlusion with exercise	After occlusion with exercise		
1	5·18	5·36	+0·18	+3·5
2	4·29	4·50	+0·30	+7·1
3	6·09	6·12	+0·03	+0·5
4	3·41	3·49	+0·08	+2·3
5	5·10	5·16	+0·06	+1·2
6	3·51	3·52	+0·01	+0·3

TABLE 2. Serum cholinesterase activity of cases of myasthenia gravis under prostigmin treatment: (b) before and after occlusion at rest; (b) before and after occlusion with exercise

Case no.	No. of hr. since last dose of prostigmin	Dose of prostigmin in mg. and route of administration	(a) At rest, before occlusion (units)	At rest, after occlusion (units)	Difference	% difference in Q (CO_2)
7	3	2·5 subcutaneously	2·77	2·69	−0·08	− 2·9
8	10	15 oral	4·80	4·84	−0·16	− 3·3
9	24	15 „	7·22	6·99	−0·23	− 3·2
10	12	15 „	3·93	4·40	+0·42	+10·6
11	12	15 „	5·77	5·33	−0·44	− 7·6
12	24	15 „	4·27	4·71	+0·44	+10·3
13	24	15 „	3·57	3·54	−0·03	− 0·8
14	24	15 „	3·59	3·37	−0·22	− 6·1
15	5	1·5 subcutaneously	5·02	4·88	−0·14	− 2·7

			(b) At rest, before occlusion with exercise	After occlusion with exercise		
7	3	2·5 subcutaneously	2·30	2·45	+0·15	+6·5
8	10	15 oral	3·39	3·20	−0·19	−5·6
9	24	15 „	7·84	7·46	−0·38	−4·8
10	12	15 „	4·23	4·05	−0·18	−4·3
11	12	15 „	4·94	4·63	−0·31	−6·3
12	24	15 „	3·50	3·31	−0·19	−5·4
13	24	15 „	3·51	3·49	−0·02	−0·6
14	24	15 „	5·67	5·51	−0·16	−2·8
15	5	1·5 subcutaneously	4·60	4·74	+0·14	+3·0

TABLE 3. Serum cholinesterase activity of cases of myasthenia gravis not under any treatment: (a) before and after occlusion at rest; (b) before and after occlusion with exercise

Case no.	(a) At rest, before occlusion (units)	At rest, after occlusion (units)	Difference	% difference in Q (CO_2)
16	2·97	3·11	+0·14	+4·5
17	1·92	2·04	+0·12	+6·2

	(b) At rest, before occlusion with exercise	After occlusion with exercise		
16	3·27	3·37	+0·10	+3·06
17	2·38	2·31	−0·07	−2·9

DISCUSSION

In normal individuals, in the control series of occlusion without exercise, there was no apparent variation in the individual levels during a period of occlusion of the circulation for 4 min. Nor was there any significant difference in the serum cholinesterase level as a result of muscular exercise. While our observations were made during occlusion of the circulation, and are not directly comparable with those of Hall & Lucas [1937], and Richter & Croft [1942], our

conclusions are in the main in agreement with the conclusions of the former workers. This suggests that the cholinesterase level of the serum in normal individuals is relatively stable, and that it does not alter under normal physiological conditions.

In the control observations, occlusion of the circulation without exercise produced an increase of 10 % in the cholinesterase activity of the serum in two of the patients with myasthenia gravis who were under prostigmin treatment (Table 2). In the experiment with exercise during occlusion of the circulation there was no significant change in the serum cholinesterase activity of any of the patients in this group as a result of the exercise.

The untreated patients with myasthenia gravis (Table 3) showed no significant change in the cholinesterase activity of their serum as a result of occlusion of the circulation, with or without exercise.

It would thus appear that, as far as the cholinesterase activity of the serum is concerned, the myasthenic person, whether under treatment with prostigmin or not, responds to exercise during occlusion of the circulation in a manner similar to that of the normal individual.

SUMMARY AND CONCLUSIONS

The effect of exercise during occlusion of the circulation, on the serum cholinesterase activity was observed in 6 normal adults, 9 patients with myasthenia gravis who were under prostigmin treatment and 2 untreated patients with myasthenia gravis.

In normal persons and in patients with myasthenia gravis (whether under prostigmin treatment or not), no significant change in the serum cholinesterase activity was observed as a result of muscular exercise during occlusion of the circulation.

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THE EFFECT OF TEMPERATURE ON BLOOD FLOW AND DEEP TEMPERATURE IN THE HUMAN FOREARM

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The plethysmographic method of Lewis & Grant [1925] for measuring blood flow in the human forearm involves placing the limb in water at a constant temperature to avoid the effect of temperature change on the blood flow. The water temperature chosen by various authors has varied from 45° C. [Prinzmetal & Wilson, 1936] to 14° C. [Abramson, Katzenstein & Ferris, 1941], but temperatures ranging from 30 to 37° C. are more usually employed [Lewis & Grant, 1925; Grant, 1938; Abramson, Zazeela & Marrus, 1939]. Using this latter temperature, various workers have found that after the first 30 min. the blood flow remains fairly steady with slight irregular fluctuations [Grant, 1938; Abramson *et al.* 1939]. The blood flow at higher temperatures has not been carefully studied. Freeman [1935] made an extensive study of the effect of temperature on the circulation in the hand, but there is no comparable work on the forearm.

The present paper describes the variations in blood flow as a result of changes in the temperature of the surrounding water. The results have been obtained under standard conditions from a group of healthy subjects. Deep muscle temperature has also been investigated. Lefèvre [1911] found that immersing the human limb in water-baths at temperatures ranging from 5 to 35° C. had no effect on the temperature 1 cm. below the fascia. Grant & Pearson [1938], with the arm cooling in air at 15° C., found that the deep muscle temperature fell 4·7° C. in 75 min. Macleod, Self & Taylor [1920] found a rise in temperature in the gluteal muscles of the rabbit when hot pads were applied to the overlying skin, and Macleod & Taylor [1921], also using the rabbit, obtained a fall of 4° C. in muscle temperature, 1·5 cm. below the surface, after 1 hr. exposure at 15° C. Contrary to Lefèvre, we have found considerable changes in deep muscle temperature when the limb is placed in water-baths at different temperatures, and these facts are related to many phenomena in physiology, biochemistry and medicine.

METHODS

Forearm temperature was varied by altering the temperature of the bath in which it was immersed. Forearm blood flow was measured plethysmographically by the method of Lewis & Grant [1925]. Our procedure differed from the original in a few points. A watertight forearm fit was made by closing each end of the plethysmograph with a detachable 0·25 in. thick Admiralty rubber sheet diaphragm with a central hole carefully cut to fit the contour of the subject's forearm nicely but not tightly. The diaphragms were firmly secured to the ends of the plethysmograph by wing nuts and bolts which pierced successively 1 in. flanges on the ends of the plethysmograph, the rubber diaphragms, and detachable brass plates. Each plate had a central hole large enough for a big forearm. The plate at the lower end of the plethysmograph was cut into two halves, to enable the hand to go through. Besides squeezing the diaphragms against the flanges the plates prevented them from bulging outwards, though the thickness of the rubber was probably sufficient to have prevented this in any case. The diaphragms were secured to the skin by thin sheet rubber cuffs 2 in. long fixed to the skin with rubber flooring cement. In a few experiments the cuffs were replaced by a short 'made to measure' sleeve of the same material attached above and below round the holes in the diaphragm [cf. Grant & Pearson, 1938]. In either case a watertight fit was obtained, and successive 2 c.c. increments in the water content of the plethysmograph were each accompanied by an equal movement of the writing point of the float recorder. To fit the plethysmograph to the forearm the components were put on separately and then bolted up.

The opening in the plethysmograph through which variations in forearm volume were transmitted to the recording system was 1 in. in diameter. The vertical glass tube above it was 1·25 in. in diameter. The basic level of the water in the vertical tube was adjusted to be about the same as that of the water in the bath in which the arm and plethysmograph were immersed; at the higher temperatures used the level sometimes had to be readjusted because the forearm swelled. Abramson *et al.* [1939] show the importance of these points.

Thirty seconds before a tracing of the flow was taken the circulation through the hand was arrested by throwing an air pressure of about 200 mm. Hg into a sphygmomanometer cuff round the wrist [Grant & Pearson, 1938]. Time spent calculating the flows from the tracings was saved as follows. For each experiment a time constant T was calculated from the formula

$$T = \frac{60}{V \times D},$$

where V is the volume of the forearm contained in the plethysmograph in 100 c.c., and D is the vertical distance, in cm., described by the writing point of

the float recorder per 1 c.c. increase in the fluid content of the plethysmograph. To find the blood flow from any given record a sloping line was drawn through the sloping part of the tracing recorded after application of the collecting pressure and produced to cut the horizontal line of the seconds time marker. A horizontal distance equal to T sec. was measured along the time tracing from the point at which it was cut by the sloping line. The vertical distance from the point thus obtained to the sloping line was measured with a cm. rule, and the figure obtained was the blood flow in c.c./100 c.c. forearm/min.

Forearm deep temperature was taken in the depths of the dorsal ante-brachial muscles near the periosteum of the lateral surface of the radius about 1 in. below the head of the bone. A standardized thermocouple galvanometer circuit was used [Lewis, 1924]; the test junction lay about 1 mm. from the point of a fine hypodermic needle. The relative accuracy of the readings was about 0·1° C., and the absolute accuracy about 0·25° C.

Experimental technique

Approximately seventy experiments were carried out on five normal men aged 25–40. The blood flow and deep muscle temperature were measured in one forearm which was immersed in water kept at a constant temperature throughout the experiment. The temperatures employed were 13, 20, 25, 30, 32, 35, 38, 40, 42·5, and 45° C.

The plethysmograph was fastened into position on the arm; this took from 7 to 15 min. The subject then reclined on a couch with his head and shoulders raised, so the arm could comfortably be placed in a water-bath lying by his side. In this position the forearm lay at the same level, or just below, the heart. The needle carrying the thermojunction for measuring the deep muscle temperature was then inserted through the skin and fleshy part of the brachioradialis muscle, and the arm and plethysmograph immersed, to a point just above the elbow, in water at the chosen temperature. The plethysmograph was filled with water at the same temperature; the sphygmomanometer cuffs were placed on the arm and the wrist, and the first reading taken. A note was made of the forearm used, of the time when it was first exposed, and the time it was immersed in water. Observations were made at 5 min. intervals of the following: deep forearm temperature, forearm blood flow, water-bath temperature, water temperature in the plethysmograph. Room temperature, which varied from 15 to 20° C., was noted at approximately 15 min. intervals; pulse rate, blood pressure, and mouth temperature were noted at regular intervals in many of the experiments. The water-bath temperature was kept constant throughout the experiment to within a few tenths of a degree. The duration of each experiment was usually 2 hr., but in several cases more extended observations were made. In one subject, the effects of several of the water temperatures were examined in one sitting; in one case three tem-

peratures below 37° C., and in another four temperatures ranging from 38 to 45° C. Each water temperature was kept constant for the usual 2 hr. and then the temperature was changed to the new level. Prolonging the experiment
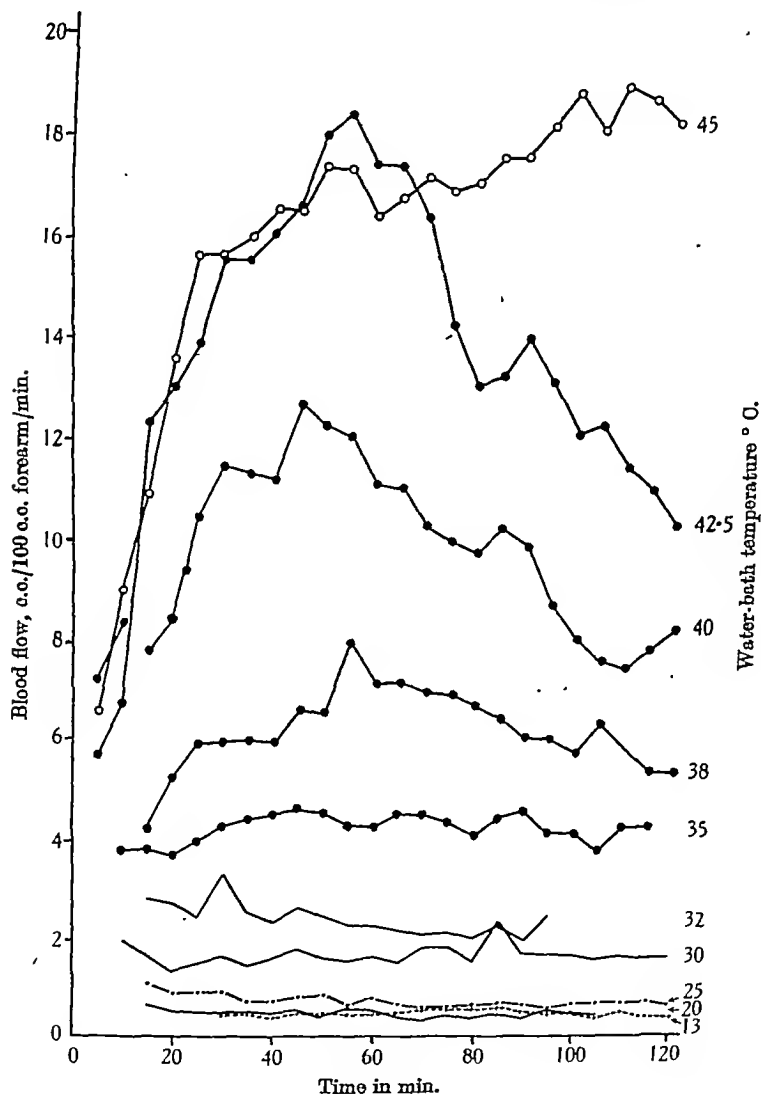
Fig. 1. Average forearm blood flow at different water-bath temperatures, five subjects. The arm was placed in the water at time 0. Note the up and down character of the flow at 38, 40 and 42·5° C. and the sustained flow at 45° C.

had no adverse psychological effect. The seventy experiments were spread over a considerable time, so that observations were made at all the seasons of the year.

RESULTS

In Fig. 1 is shown the average blood flow expressed in c.c./min./100 c.c. forearm, for the same five individuals at different water-bath temperatures. The curves shown for water-baths of 20 and 32° C. are the averages of three individuals only. The data for the first 15 min. in the water-bath are incomplete, owing to the time taken completing preparations for recording the
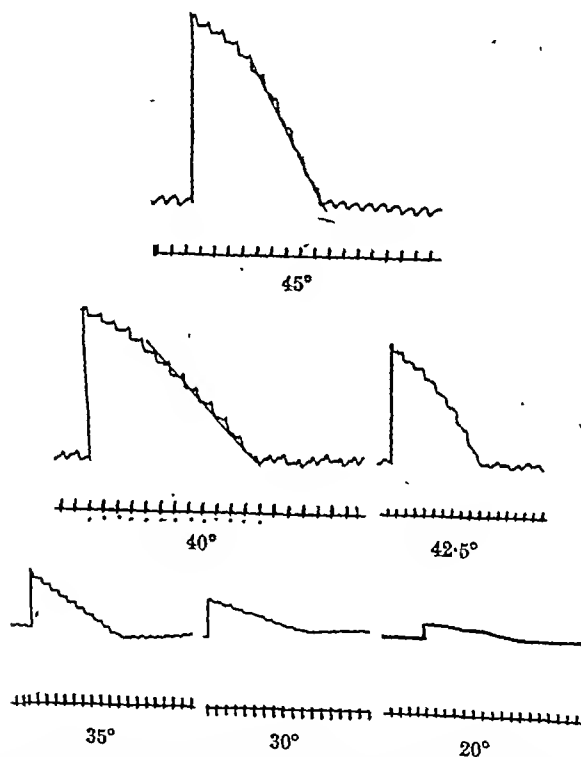
Fig. 2. Subject O. G. E. Each tracing was recorded approximately the same time after immersion of the forearm in water at the temperature indicated. Time in seconds.

flow. Extrapolation of the curves suggests that at time 0 (time arm placed in bath) the flow would have been about 3–4 c.c./min./100 c.c. forearm. Water colder than 35° C. has a constrictor effect with reduction in blood flow. An increased blood flow and vaso-dilatation is produced by water at a higher temperature than 35° C. At the highest temperature employed these flows were very considerable; in one subject at 45° C. the blood flow reached 28 c.c./min./100 c.c. forearm. In Fig. 2 individual flow tracings obtained from one subject at different temperatures are shown.

The flow-time relations can be considered under three heads:

(a) 13–35° C. After the first 15 min. the flow remains almost constant. There is a slight decrease on the average over the 2 hr., as shown in Table 1. Fluctuations in the individual curves from one reading to the next are small.

TABLE 1. Blood flows, averages of five individuals, in water-baths below body temperature. The flows are given in 30 min. averages, ignoring the flows for the first 15 min. The periods are indicated in terms of time in minutes after immersion

Bath temp. °C.	Blood flows (min.)			
	15–45	45–75	75–105	105–end
35·0	4·1	4·4	4·25	4·10
32·0	2·65	2·25	2·1	2·1
30·0	1·55	1·52	1·75	1·6
25·0	0·86	0·72	0·6	0·65
20·0	0·46	0·43	0·54	0·5
13·0	0·52	0·48	0·4	0·33

(b) 37–42·5° C. The flow increases steadily and rises to a maximum, the time of the peak varying from one individual to another, but averaging 1 hr. from the beginning of the experiment. The flow then steadily diminishes during the next hour. This rise and fall has been found in all individuals examined at these temperatures. The 'up and down' character of the blood flow increases with rise in water-bath temperature, and it varies in degree with different individuals. In some it is prominent at 37° C., in others it is only at a higher temperature that the up and down is conspicuous. The peak flow occurs earlier on the average the higher the temperature; at a water-bath temperature of 38° C. the average peak time was 70 min., and at 42·5° C. this was reduced to 55 min.

(c) At 45° C. the blood rises rapidly to a maximum within 30 min., but instead of dying away, as in the temperature range 37–42·5° C., the flow remains almost constant at this maximum, or may slightly increase over the 2 hr. In all subjects examined there is a striking contrast in the blood flow at this temperature and at the lower temperatures.

An important point established by the above result is that at 37–42·5° C. the blood flow does not stabilize, at least not within 2 hr. Some experiments were carried out for much longer periods at 37° C. In Fig. 3 is shown the blood-flow curve for one individual at this temperature over a period of 5 hr. After the preliminary rise and fall there are no further major fluctuations.

In most experiments a fluctuation in flow from one reading to the next was found. These variations do not appear in Fig. 1, as they are smoothed out by averaging five different curves. In Fig. 3 the flow for an extended period for one individual is shown, together with a flow for 3 hr. at 42·5° C. Here the fluctuations are clearly seen. They have been described by others, including Abramson et al. [1939], who stated that these fluctuations were less marked at 45° C. than at lower temperatures. The interval between the crests of

fluctuations is variable, from 10 to 45 min. The degree of fluctuation also varies considerably from one individual to another. In view of Abramson's observation that these waves are less prominent at higher temperatures, an attempt has been made to estimate their frequency at different temperatures in different
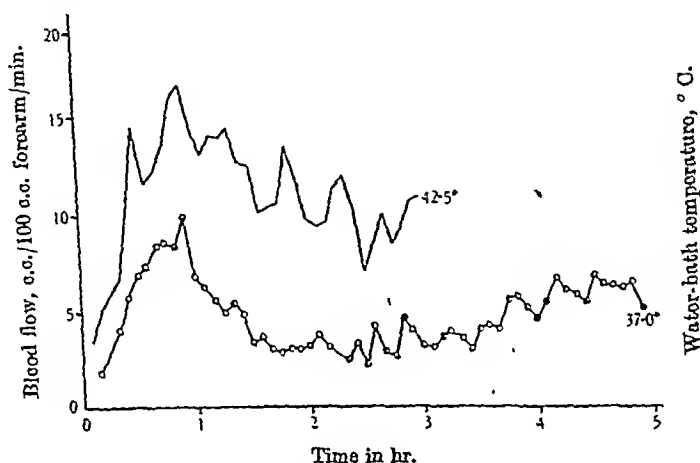
Fig. 3. Subject A. S. E. Shows the fluctuations in flow, more marked at the higher temperature, and the absence of any further major deviation after the up and down in the lower curve.

individuals. This was done by drawing a smooth curve over the actual curve obtained in individual experiments, and the degree of fluctuation from this smooth curve estimated. Minor fluctuations were ignored, only fluctuations of

TABLE 2. Degree of fluctuation of blood flow at various water-bath temperatures

Subject	Water-bath temperature			
	45.0° C.	42.5° C.	40.0° C.	38.0° C.
N. C. H.	7	7	3	1
O. G. E.	8	8	5	1
A. S. E.	13	7	4	4
T. F.	6	6	5	1
H. B.	9	6	4	5

more than 1 c.c. were counted. Table 2 shows the results obtained, the degree of fluctuation increasing with rise of temperature, so we find that fluctuations at 45° C. are more numerous than at any lower temperature.

The constancy of flow was also investigated. Flows were measured in the same individual at the same temperature on different occasions, and in some cases these measurements were spread over as long as a year. Averaging the flows and estimating the standard error showed that the greatest variation occurred during the earlier part of the flow measurement. After the first hour the standard error decreased, and by the end of 2 hr. flows were strikingly constant. The variation chiefly affected the peak flows and was more marked

at higher temperatures. As there may be many minor factors affecting the flow, such as seasonal differences, four flows at 42·5° C. were measured on the same individual over a period of 14 days. The results are shown in Fig. 4. Once more the peak flow is variable, but for the last 60 min. the flows are closely similar.
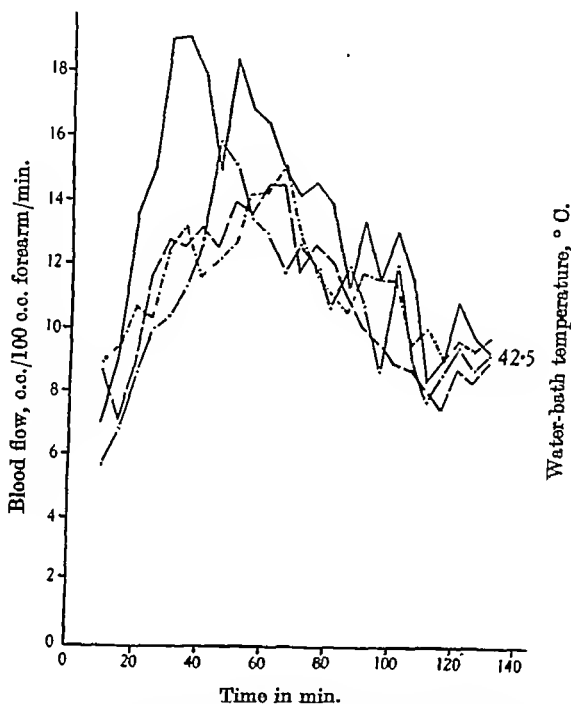
Fig. 4. Subject O. G. E. Four blood-flow curves each after the forearm had been immersed in water at 42·5° C. Shows the close agreement between the readings during the last hour.

It was possible during the long series of experiments to observe the effect of sleep. Ingram [1936] found that cutaneous vaso-dilatation followed sleep, and we have observed that a rise in skin temperature together with a definite diminution of blood flow coincided with the onset of sleep, or occurred within a few minutes. This diminution of flow was maintained for approximately 15 min. and then frequently increased again to the previous value. Abramson *et al.* [1939] observed this diminution in flow in the hand.

The average forearm blood flow

Valuable inferences regarding the circulation have been based on a comparison of the normal average forearm flow with that found in abnormal conditions, such as after sympathectomy [Grant & Holling, 1938], in hyperthyroidism [Abramson & Fierst, 1941] and in shock [Freeman, Shaw & Snyder,

TABLE 3. Comparison of figures for blood flow obtained by previous workers (column B) and in the present investigation (column A). The flows are given in c.c./100 c.c. forearm/min.

Bath temp. °C.	Blood flow		Room temp. °C.	Author
	A	B		
10–15	0·5		17	
		0·7	27	Abramson *et al.* [1939]
20·0	0·5		17	
24·0		1·65		Prinzmetal & Wilson [1936]
25·0	0·7		17	
30·0	1·6		17	
		1·5	15	Grant & Pearson [1938]
32·0	2·3		17	
		1·5–2·5	27	Abramson *et al.* [1939]
35·0	4·3		17	
37·0	5·9		17	
		5·0	22–26	Kunkel, Stead & Weiss [1939]
45·0	17·6		17	
		8·9		Prinzmetal & Wilson [1936]
		15·0	27	Abramson *et al.* [1939]
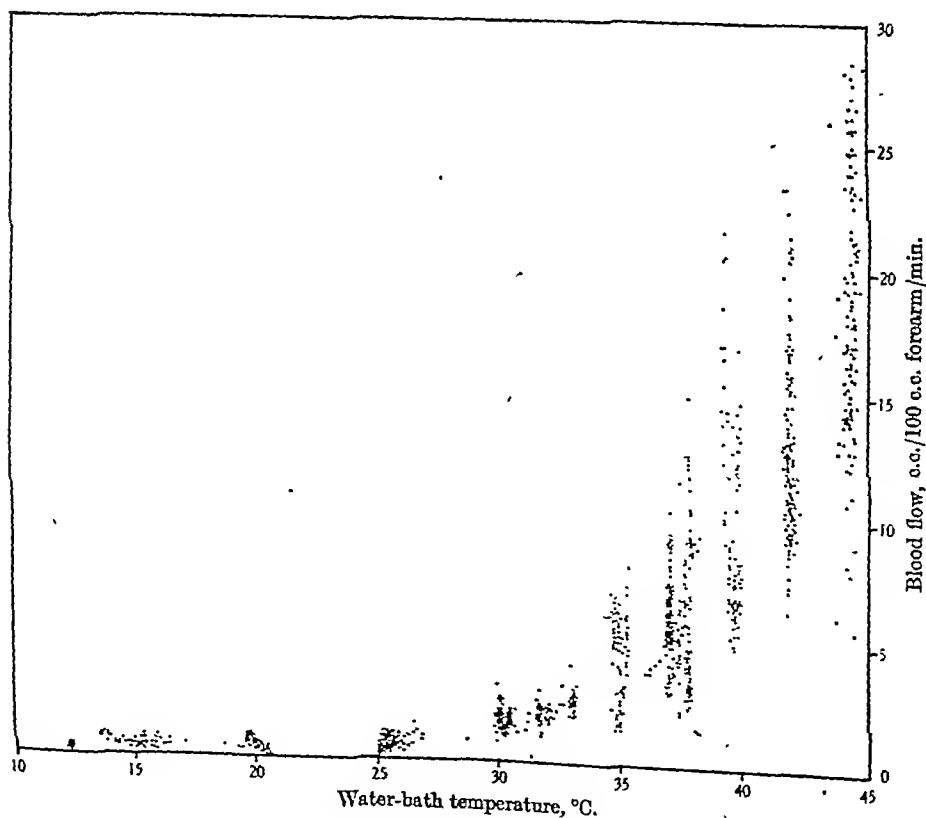
Fig. 5. All blood-flow readings on all subjects at all water-bath temperatures.

1936]. The large number of blood-flow readings obtained during this investigation enable us to calculate the average flow at various water-bath temperatures. The readings taken during the first 30 min. of the flows have been neglected: as shown above this is the period of greatest variability, and the initial flows may be affected by previous muscular activity.

The average of the 5 min. readings of the flows from the end of 30 min. in the water-bath up to the end of the second hour are shown in Table 3, in which the averages obtained by other workers are also given. The averages for flows at water temperatures of 38, 40, and 42·5° C. are not included, since at these temperatures the flow exhibits a rise and fall over the period of 2 hr. Comparing our figures with those obtained by other workers, there is a fair measure of agreement. This is the more striking when the different conditions that obtained are considered. Abramson and his colleagues, for example, worked at an average room temperature of 27° C., as opposed to the average room temperature of 17° C. employed by English workers.

In Fig. 5 the individual flow readings have been plotted against water-bath temperature. It will be seen that there is a fairly abrupt increase of blood flow at approximately body temperature. The average flows below 37° C. are very nearly the same in all individuals examined. At higher temperature there is considerable variation from one individual to the next. At 45° C. the average figures for five different subjects are 11·0, 14·2, 17·6, 19·2 and 25·4 c.c./min./100 c.c. forearm.

Deep muscle temperature

The deep muscle temperature depends on at least five factors: (1) the water-bath temperature, or environmental temperature; (2) the length of the experiment; (3) the rate of blood flow; (4) the body temperature; (5) the local metabolic rate.

Water-bath temperature. Fig. 6 shows the average deep muscle temperature of the five subjects at 5 min. intervals for 2 hr. after immersion at the given temperature. The point at which the temperature was measured, about 1 in. below the skin covering the brachioradialis muscle, is not far from the middle of the thickness of the forearm. When the water-bath temperature equals body temperature heat is not lost or gained from the arm, and the deep muscle temperature reaches an equilibrium point equal to body temperature or slightly above it owing to the local metabolic production of heat. At higher bath temperatures the rise in muscle temperature becomes gradually less with each increase in bath temperature. The average deep muscle temperature in baths at 42·5 and 45·0° C. is identical, 39·0° C. In fact, in some individuals the deep muscle temperature actually decreases on raising the bath from 42·5 to 45·0° C. At all bath temperatures above body temperature the deep muscle temperature is lower than that of the bath.

At bath temperatures below 37·0° C. the deep muscle temperature is invariably higher than that of the bath, from 32·0 to 20·0° C. being approximately 2·5° C. above; at lower bath temperatures there is a greater difference between bath and muscle temperature, being from 4 to 5° C. higher with a water-bath of 13° C. When the arm is immersed in these cold baths, the sensation of cold only persists for some 10 min. There is no discomfort, but when the arm is taken out of the bath after 2 hr. or more muscular movements are difficult to perform and are very slowly executed [Lewis, 1941]. Generalized
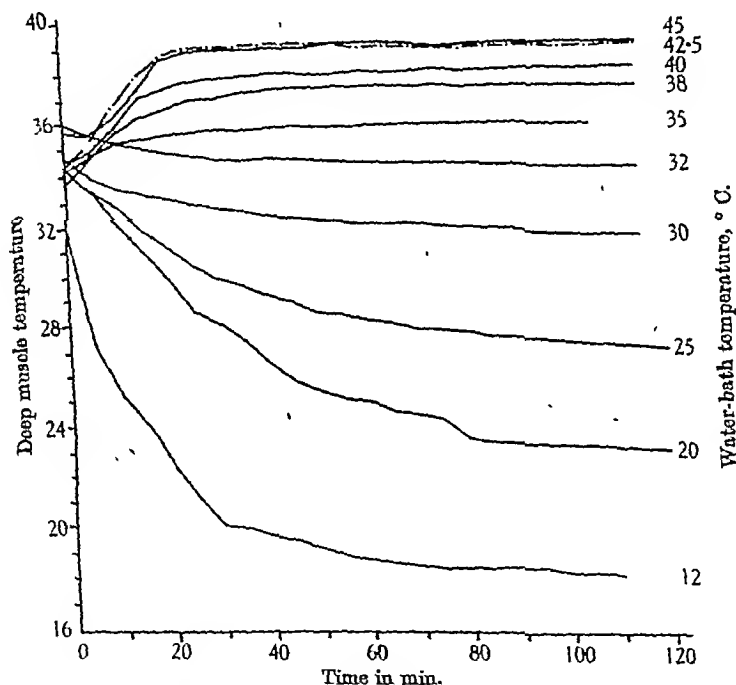
Fig. 6. Average deep muscle temperature at different water-bath temperatures, five subjects. The arm was placed in the water at time 0.

shivering is not induced. The hot baths are comfortable up to a temperature of 42·5° C., but 45·0° C. is about as hot as can be tolerated by the average individual. An attempt was made to estimate the flow at a higher temperature, but this was found to be impossible because of the pain, which is felt mainly in the fingers. These hot arm baths induced sweating.

Length of the experiments. It takes a considerable time for the deep muscle temperature to come into equilibrium with the water-bath temperature, particularly in cold baths. In water-baths below 35·0° C. the deep tissues cool continuously for at least 1 hr. before coming into approximate equilibrium with the bath temperature. The equilibrium time steadily increases with lower

water-bath temperature, and after 2 hr. in a water-bath at 13° C. the deep muscle temperature may still be falling slightly. Water-baths above body temperature raise the deep muscle temperature, but equilibrium is reached more quickly from 40 to 60 min. after immersion. Water-baths at body temperature also affect the deep muscle temperature, because this has usually cooled during the application of the plethysmograph, so at all bath temperatures it takes a considerable time for the deep tissues to come into approximate equilibrium with the bath temperature. It is clear that the temperature throughout the whole thickness of the forearm is dependent upon that of the water surrounding the limb, since the deep temperature is initially approximately the same in all experiments and diverges within 10 min. after immersion.

Blood flow. It was stated above that the deep muscle temperature may actually fall on raising the water-bath temperature from 42·5 to 45·0° C. This is due to the greatly increased blood flow at the higher temperature. The temperature of the blood entering the arm is not higher than 37·0° C. and so a rapid blood flow will cool the arm and prevent a greater rise of deep temperature. The very rapid fall of deep muscle temperature when the arm is placed in a cold bath is due to the greatly decreased blood flow, which at bath temperature of 13–20° C. may sometimes be almost imperceptible.

Body temperature. The body temperature affects the deep muscle temperature by altering the temperature of the blood entering the arm, and body temperature is affected by the water-bath temperature. Under the experimental conditions adopted, the subject is lying at rest on a couch for at least 2 hr. During this time, even without the arm in a water-bath, there is a gradual progressive fall of body temperature, decreasing pulse rate and a slight fall of blood pressure, as the subject approaches a basal metabolic rate. The water-bath into which the arm is placed will either accentuate or diminish this fall of body temperature. With cold baths the increased fall in temperature is not marked, because the blood flow is very small. But when the water-bath is raised above 37·0° C. there is a sharp rise of body temperature, as the large volume of blood flowing through the arm is heated. In each individual the body temperature differs by approximately 1° C. with the arm in the coldest and hottest baths employed. In hot baths there is an initial rise of body temperature, a plateau and a gradual fall. In cold baths there is a steady fall.

Metabolic production of heat in arm. The effect of local metabolism on tissue temperature could not be measured directly and has not been further considered.

DISCUSSION

Grant [1938] described a very gradual diminution in blood flow which sometimes occurred after the first 30 min. using a water-bath of 30° C. This can be also seen in our curves at 32 and 25° C. in Fig. 1. Grant tentatively suggested

in a footnote that the explanation might be a progressive diminution in the concentration of a circulating adrenaline-like substance, used up during the prolonged rest. As Fig. 6 shows, the temperature of the muscle falls progressively for a very long time and the effect seems probably due to a gradual cooling.

We have found no suggestion of an increased flow in cold baths, although Rein & Schneider [1930] found an increased flow in dogs' limbs, when the animals were exposed to a cold blast of air. This often induced shivering, which may account for the different finding.

From the theoretical standpoint the main new fact is the behaviour of the blood flow when the forearm is in moderately hot water (37–42·5° C.). We are at present unable to explain why the flow should diminish progressively during the second hour; or why it does not do so in water at 45·0° C. The following possible factors have been considered. Mechanical obstruction due to oedema of the arm is not responsible, since the die away does not occur at 45·0° C. when any possible oedema would be more marked. Herrick, Essex, Mann & Baldes [1934] found an increased blood flow in the hindlimbs of dogs after a meal and persisting from 2 to 6 hr. However, we have not found any relation between the taking of a meal and the presence of the die away, which occurs both when the flow is measured immediately after a meal and after a 12 hr. fast. Changes in blood pressure are unlikely to be of importance, as we have found only a trivial alteration during our experiments.

The die away can also occur in a sympathectomized limb, but in one case of sympathectomy we found a marked die away in the normal limb, and only a slight die away in the sympathectomized arm. The die away can occur, in our experience, in the absence of the sympathetic, but it is possible that the sympathetic nervous system plays some part in the production of this phenomenon. That the fall might be due to the gradual fall of the general metabolic level towards basal conditions seemed possible. But we have found that after a prolonged rest on the couch for 3 hr., the subsequent flow with the arm in water at 42·5° C. for the next 2 hr. exhibited a typical rise and die away. Grant [1938] has suggested the diminution of a circulatory adrenaline-like substance. If there is such a substance it did not diminish during the preceding rest. One experiment we have carried out suggests the possibility of a vasodilating factor being washed out from the arm during an increased flow. The arm was placed in water at 38° C. for 2 hr., and then the water-bath was heated to 40·0° C. and the flow measured for the next 2 hr. On this occasion the die away was very slight, the flow not reaching a marked peak but remaining fairly constant. The same subject has been repeatedly examined at the same temperature and at 42·5° C., and on all other occasions has shown a well-marked rise and die away of blood flow. It is therefore possible that the preliminary heating of the arm for 2 hr. washed out a vaso-dilating substance.

Natus [1910] found that the blood vessels of the isolated rabbit's ear responded to local heat by vaso-dilatation which was followed by a partial recovery of tone. This experiment suggests that the die away is possibly a local effect.

From the point of view of the use of the forearm flow method in attacking circulatory problems it is evident that, if for some reason the forearm is to be surrounded by water between body temperature and 42·5° C., the normal rise and fall for the first 2 hr. must be taken into account, both in planning the experiment and in interpreting the results. It is of interest in this connexion that we found a fairly constant flow with the arm immersed in water at 37·0° C. from the end of the second to the end of the fifth hour (Fig. 3). There was no evidence that continuing the flow recording for this period was upset by psychological disturbances.

The contrast between flows up to 42·5 and 45·0° C. is difficult at present to understand. Lewis & Love [1926] described the reflex flush of the skin produced by water-bath of 43–44° C. This reflex flush they considered to be indicative of the release of histamine, the first stage in tissue damage. It is not possible to say if there is any connexion between these two observations, except to point out that a change in the character of the blood flow appears to coincide with the first signs of tissue damage due to heat.

Since the forearm contains about 60 % muscle [Abramson & Ferris, 1940], it is generally assumed that muscle flow contributes substantially to that measured by the plethysmograph. It seems almost certain that part of the increase in flow which occurs at high temperatures must be traversing muscle. Moderate heat is known to cause vaso-dilatation in blood vessels in brain [Florey, 1925], pancreas [Natus, 1910], kidney, liver spleen, the isolated extremity, and in the rabbit's ear (for references see Lewis [1929]). Since the forearm muscle temperature, even near the middle of the limb, is influenced by the temperature of the water surrounding the limb, it is almost certain that the blood flow is equally affected. Other considerations also support this conclusion. The volume of skin in the forearm is approximately 13 % [Abramson & Ferris, 1940]. The resting blood flow is approximately 4 c.c./100 c.c./min.; in the bath at 45·0° C. flows of 28 c.c. have been recorded. If it is assumed that the whole of the extra flow was passing through the skin, this would give a figure of 184 c.c./min./100 c.c. skin. Wilkins, Doupe & Newman [1938] found a peak flow of 120 c.c./min./100 c.c. finger. This was for the two terminal phalanxes only, and they state that the blood flow decreased in the middle phalanx, being one-third of that in the terminal phalanx. For the whole finger, Grant & Pearson obtained a flow of 30 c.c., indicating a diminishing blood flow in the finger from the terminal phalanx proximally. The finger contains approximately 50 % skin [Wilkins et al. 1938], which gives, on Grant & Pearson's findings, a flow equal to 60 c.c./100 c.c. skin. The blood flow in the

whole hand is greater per volume than that of the forearm [Abramson & Ferris, 1940], so a figure of 184 c.c./100 c.c. forearm skin is absurd. It is therefore reasonable to conclude that a considerable proportion of the increased blood flow at high temperatures in the forearm traverses the muscle.

In hot baths the skin temperature will be raised more rapidly than the deep muscle, and cutaneous vaso-dilatation should also be more rapid. But the blood flow climbs comparatively slowly, roughly parallel to the rise in deep muscle temperature, or lagging slightly behind. This observation also supports the view that the extra blood flow involves muscle vessels as well as skin.

SUMMARY

The blood flow and deep muscle temperature have been measured in the human forearm for 2 hr. after its immersion in water at temperatures ranging from 13 to 45·0° C.

The average forearm blood flows range from 0·5 c.c./100 c.c. forearm/min. at 13·0° C. to 17·6 c.c. at 45·0° C.

The flow time relations fall into three groups:

(a) 13–35·0° C. Slight decrease in flow during the 2 hr. Not conspicuous except in first 15 min.

(b) 37–42·5° C. The flow increases to a maximum in about 1 hr., then decreases steadily.

(c) 45·0° C. Increases to a maximum in 30–45 min., then remains constant.

The significance of these time relations is discussed.

The higher the water temperature the more frequent are the spontaneous fluctuations in blood flow.

The deep muscle temperature not far from the middle of the thickness of the upper part of the forearm ranged from 18·0° C. after 2 hr. immersion at 13·0° C. to 39·0° C. after 30 min. immersion at 42·5–45·0° C.

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ON SYMPATHETIC VASOCONSTRICTOR TONE
IN HUMAN SKELETAL MUSCLE

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Bülbring & Burn [1935, 1937] have shown that the sympathetic nerves to the skeletal muscles in dogs, cats, hares, rabbits and monkeys have a vasoconstrictor component. Anrep, Blalock & Samaan [1934] and Baetjer [1930] have found sympathetic vasoconstrictor tone in resting dog and cat muscle. In man the position is not clear. Sympathectomy is sometimes performed in the expectation of releasing constrictor tone in muscle and so quickening blood flow. But evidence that it would do so seems lacking, for Woollard & Phillips [1932], Friedlander, Silbert, Bierman & Laskey [1938] and Grant & Holling [1938] blocked human motor nerves but could not detect any rise in temperature in or over the muscles.

EXPERIMENTAL

Gilding [1932] showed that the sympathetic fibres to cat's muscle travel in the mixed peripheral nerves; he reviews the literature. Our method was based on the assumption that, if such fibres existed in man, they would reach their destination by the same route. If so, just above the elbow, the sympathetic to the musculature of the forearm would be in the radial, median and ulnar nerves deep to the deep fascia. At this level the sympathetic to the forearm skin, which accompanies the cutaneous nerves [Lewis, 1929], would be in the subcutaneous tissue in the antebrachial cutaneous nerves. Therefore blocking the deep nerves would leave the sympathetic path to the forearm skin intact but would release any sympathetic vasoconstrictor tone there might be in the forearm muscles; the significance of the vasoconstrictor tone in the muscle could be judged by the change in the forearm blood flow as shown by Lewis & Grant's [1926] plethysmographic method.

Comparison of the forearm flows before and after blocking would have been open to the objection that a difference might have been the result of a variation in the blood pressure or circulating vasomotor substances. Therefore, after

blocking, the flow in the paralysed forearm was compared with that in the opposite normal one.

The temperature of the forearm muscles was kept at about its normal level by immersing the limbs in water at 35° C. [Barcroft & Edholm, 1943]. Experimental and natural release of tone would then be expected to increase forearm muscle flow by about the same extent.

METHODS

The subjects were healthy men, aged 20–40. Room temperature was about 18° C. The technique for blocking the deep nerves was developed by W. B. The local anaesthetic was 4 % procaine and 1 in 50,000 adrenaline, the solution used by Woollard & Phillips [1932].

The subject lay down to avoid a feeling of faintness apt to occur during blocking of the nerves if seated. Except in very thick arms the radial nerve on the left (test) side was located by rolling it under the finger near the junction of the upper and middle two-thirds of a line joining the insertion of the deltoid to the lateral epicondyle of the humerus. The hypodermic needle was directed towards the nerve till painful tingling was felt in its peripheral distribution. After verifying that the needle was not in a vein gentle pressure was made on the plunger. If any resistance was felt the needle was withdrawn a little to avoid rupturing the nerve sheath. 2–3 c.c. of anaesthetic was then infiltrated. The median nerve was located about 1 in. above the bend of the elbow near the branchial artery and anaesthetized; then the ulnar at the back of the medial epicondyle of the humerus was similarly treated. 5–10 min. after blocking, the peripheral distribution of each nerve showed signs of sensory and motor loss. Occasionally it was necessary to infiltrate more anaesthetic.

Lewis & Grant [1926] plethysmographs were fitted to each forearm as described by Barcroft & Edholm [1943]. The subject sat up on a couch between the water-baths, and the preparations for recording the forearm blood flows were completed.

By this time the musculature below the elbow of the test forearm was usually paralysed and the hand nearly anaesthetic. Woollard & Phillips [1932] obtained little or no paralysis; we cannot explain this curious discrepancy.

Grant & Pearson's [1938] technique for inscribing the blood flows was followed. Simultaneous records of the test and control forearm flows were made at 5 min. intervals till a sequence of five similar records for averaging had been obtained. The circulation through the hands was always arrested while the tracings were being taken. $1\frac{1}{2}$–2 hr. after blocking movement and sensation began to return. The next day they were normal; there was some tenderness where the arm had been injected. In one subject there was a small area of paraesthesia which disappeared in 4–6 weeks; in this case the anaesthetic had been forced in against resistance.

RESULTS

The results are shown in Table 1 (I) and Fig. 1 (I). Fig. 2 shows a tracing.
In every experiment the test forearm was hyperaemic. The average blood flow
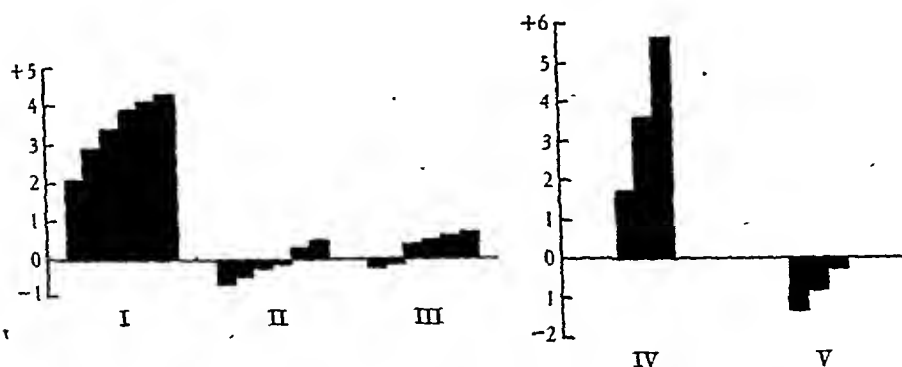
Fig. 1. The captions denote the condition of the test forearm. Ordinates: difference in test and control forearm blood flow in c.c./100 c.c. forearm/min. I, deep nerves blocked; II, normal; III, 'one' cutaneous nerve blocked; IV, skin blanched, deep nerves blocked; V, skin blanched.
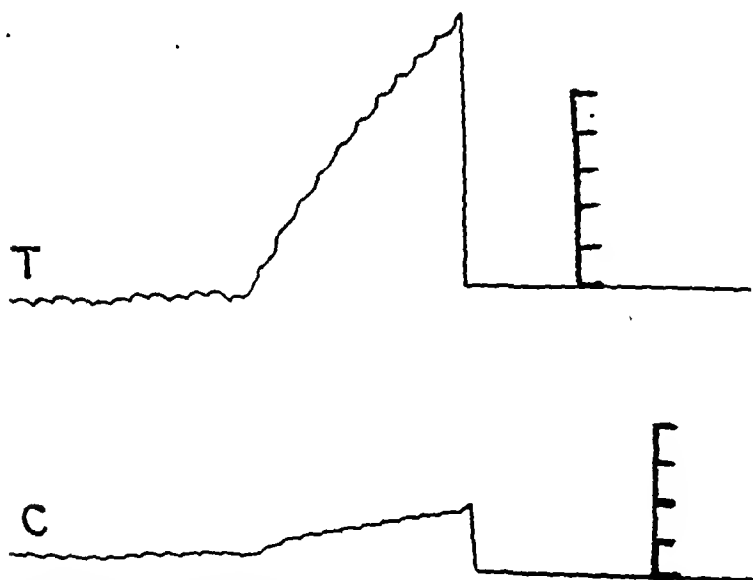
Fig. 2. Subject A. S. E. Simultaneous tracings of the blood flow in the test, *T*, and control, *C*, forearms, after the deep nerves in the test forearm had been blocked. Calibration in c.c. Water-bath temperatures 35° C. Recorded at a demonstration at the Cambridge Meeting of the Physiological Society, Oct. 1941. No seconds tracing was taken.

on the control side was 3·9 c.c./100 c.c. forearm/min., on the test side it was
7·3 c.c., an increase of 3·4 c.c. or 87%.

TABLE 1. Blood flows in c.c./100 c.c. forearm/min. in the test, *T*, and control, *C*, forearm. Water-bath temperature 35° C.

	Condition of test forearm	Subject	Test (T)	Control (C)	Difference ($T - C$)	% difference $\dfrac{100\,(T - C)}{C}$
I	Deep nerves blocked	H. B.	10·3	6·9	+3·4	+ 40
		W. B.	6·2	4·1	+2·1	+ 51
		H. B.	8·7	4·4	+4·3	+ 97
		J. S. L.	8·1	4·0	+4·1	+103
		D. C. H.	4·7	1·8	+2·9	+161
		W. B.	5·9	2·0	+3·9	+195
		Average	7·3	3·9	−3·4	− 87
II	Normal	A. S. E.	4·2	4·9	−0·7	− 14
		G. W.	3·5	3·9	−0·4	− 10
		H. B.	2·9	3·1	−0·2	− 6
		B. M. B.	2·0	2·1	−0·1	− 5
		H. M. S.	3·8	3·3	+0·5	+ 15
		W. B.	2·0	1·7	+0·3	+ 18
		Average	3·1	3·2	−0·1	− 3
III	Cutaneous nerves blocked	J. K. McC.	2·9	3·3	−0·4 (0·1)	− 12 (4)
		H. B.	7·7	8·3	−0·6 (0·2)	− 7 (2)
		P. McC.	7·5	5·5	+2·0 (0·7)	+ 36 (12)
		O. G. E.	4·4	3·2	+1·2 (0·4)	+ 37 (12)
		A. S. E.	4·8	3·3	+1·5 (0·5)	+ 45 (15)
		W. B.	3·9	2·0	+1·9 (0·6)	+ 95 (32)
		Average	5·2	4·2	+0·9 (0·3)	+ 21 (7)

The figures in brackets in III are those for one-third of the preceding figure.

Controls

(1) *Was the hyperaemia in the test forearm due to a natural difference of the circulations in the two forearms or to the nerve blocks?* Since the left forearm was always the test forearm it was conceivable, though improbable, that the results were due to the blood flow per 100 c.c. tissue on the left side being naturally greater than on the right. In six experiments (Table 1 (II) and Fig. 1 (II)) the blood flows on the two sides were recorded without blocking the nerves. The difference was negligible.

(2) *Was the hyperaemia in the test forearm due to accidental release of some sympathetic tone in the skin circulation?* In the fourth, fifth and sixth of the experiments shown in Table 1 (I), the anaesthetic seeped back along the needle track and blocked one of the three antebrachial cutaneous nerves; in the third the cutaneous nerves may have been involved. About one-third of the forearm skin in the plethysmograph was anaesthetic; its vasomotor tone paralysed. This might have been responsible for some of the hyperaemia. The significance of this was assessed as follows. All three antebrachial nerves were localized near the bend of the elbow by Trotter & Davies's [1909] method. They were then blocked. The whole of the forearm skin became anaesthetic, but there was no loss of muscular power. The blood flow in the two arms was compared and, as shown in Table 1 (III), a slight increase, on the average equal to 0·9 c.c., was obtained on the test side. The difference was not statistically significant. In five of the original experiments not more than one-third of the

sympathetic to the forearm skin was blocked, and this could not have increased forearm flow by more than 0·3 c.c. (7%) (Table 1 (III) and Fig. 1 (III)). Some other factor caused the increase of 87%.

(3) *Was the hyperaemia in the test forearm due to paralysis of tone in the skeletal muscle?* It will be remembered that the musculature below the elbow on the test side was paralysed; muscular tone must have been abolished. It was possible that the hyperaemia was due to diminished resistance to the passage of the blood through the flaccid muscle. Barcroft & Millen [1939], however, found that muscle blood flow decreased after a weak voluntary contraction relaxed—presumably because muscle metabolism and vasodilator metabolite concentration fell away, and the vascular bed shut down. By analogy paralysis of muscular tone, a weak contraction, should decrease muscle blood flow. But it was better that the question should be settled by direct experiment. This could be done by repeating the experiment on a sympathectomized forearm. The change in blood flow due to abolishing muscular tone would then be seen. Only two men with upper limb sympathectomies could be traced in Ireland. Both had had right upper thoracic ganglionectomies several years before. Their arterial blood pressures were normal, their arterial walls unhardened. In one respect their forearm circulations were unusual—there was a considerable difference in the resting flows on the two sides. Table 2 shows that the flow in F. McL.'s sympathectomized forearm

TABLE 2. Blood flow in c.c./100 c.c. forearm/min. in the sympathectomized test, T, and control, C, forearm. Water-bath temperature 35° C.

Subject	Conditon of sympathectomized test forearm	Test (T)	Control (C)	Difference $(T-C)$	% difference $\dfrac{100\,(T-C)}{C}$
F. McL.	Normal	4·1	2·8	+1·3	+46
	Deep nerves blocked	3·0	2·0	+1·0	+50
	Difference				+ 4
S. A.	Normal	2·6	3·2	−0·6	−19
	Deep nerves blocked	3·0	4·0	−1·0	−25
	Difference				− 6

was 46% greater than that in his normal one; in S. A. it was 19% less. These differences were probably due to abnormalities in the sympathectomized forearm due to the original lesion (F. McL. injury to the hand followed by severe causalgia; S. A. severe frostbite followed by violet fingers) to muscular changes due to deformity (F. M. contracture of the fifth finger; S. A. amputated index finger) and to the sympathectomy. The results are shown in Table 2. After blocking the deep nerves the flow in F. McL.'s sympathectomized forearm increased from 46 to 50% above that in his normal one, in S. A. it diminished from 19 to 25% below that in the control; negligible changes of +4 and −6%. Yet the paralysis while the flows were being recorded was absolute, and the vessels were quite able to dilate; they did so when the

temperature of the water in the bath was raised. The hyperaemia in the test forearms of the normal subjects could not have been due to loss of muscular tone.

(4) *Was the hyperaemia in the test forearm due to adrenaline vasodilation in skeletal muscle?* Grant & Pearson [1938] have shown that adrenaline vasodilates human muscle. The local anaesthetic contained a small amount of adrenaline to prolong its action; when infiltrated round the median nerve it would be quite near the brachial artery. It seemed possible that the hyperaemia in the test forearms might have been due to diffusion of adrenaline through the wall of the brachial artery into the blood going to the forearm muscles. This was tested as follows. In one experiment adrenaline was not put into the anaesthetic —vasodilation followed its infiltration round the nerves. In another 1 in 50,000 adrenaline in saline was infiltrated round the nerves—no increase in forearm flow occurred. Hyperaemia was absent in the sympathectomized forearms although the local anaesthetic contained the usual amount of adrenaline.

(5) *Was the hyperaemia in the test forearm due to release of tone in the arterial tree and passive increase in skin flow?* Claud Bernard [1876] showed that when one cervical sympathetic, in a horse's neck, was cut the pressure in the central end of the ipsilateral labial artery rose 25–60 mm. Hg. The average rise in five experiments was 43 mm. That is, when the sympathetic tone in a comparatively large artery, deep to the deep fascia, was released a rise in pressure occurred near the periphery which, had the circulation been intact, could hardly have failed to have caused a passive increase in flow through the peripheral vascular bed. Nothing definite seems to be known of the significance of sympathetic tone in the proximal branches of the human arterial tree; but, in the normal arm, Wishart [1933] found a downhill systolic pressure gradient of 20–30 mm. Hg between the radial and one of the digital arteries. The question arises whether the circulation in the forearm skin is influenced by two sets of sympathetic fibres. The first accompanying the deep nerves and acting on the branches and twigs of the brachial artery below the skin; the second accompanying the antebrachial cutaneous nerves and acting upon the vessels in the skin. If so, in the original experiments, could the sequence of events in the test forearm have been: paralysis of the deep nerves, release of sympathetic tone in the arterial tree below the skin, passive increase in cutaneous blood flow, hence the increased forearm flow? In the animal this could have been settled quite easily by repeating the experiment on the skinned limb. Fortunately, we had a method for producing what was to all intents and purposes from the viewpoint of the vascular system the human equivalent of the animal skinned limb preparation. We therefore proceeded to suppress the circulation in the skin of the test forearm, block the deep nerves and compare the blood flow on the two sides. If the test forearm was hyperaemic

PLATE 1   THE JOURNAL OF PHYSIOLOGY, VOL. 102, No. 1
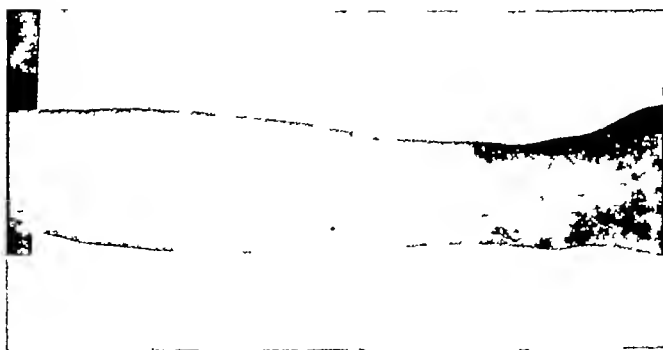
Fig. 3 a.
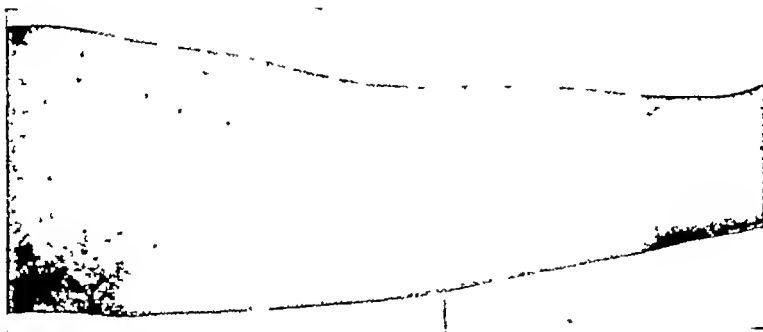
Fig. 3 b.
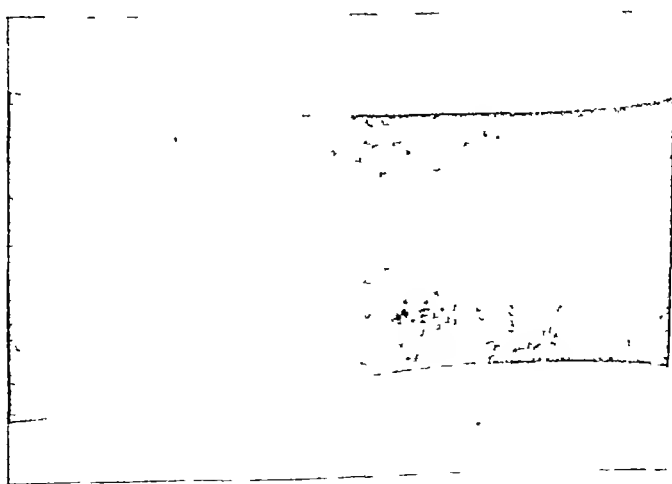
Fig. 4.

it would be proof that the nerve blocks had caused vasodilation deep to the skin. A summary of these experiments follows.

We are indebted to Prof. D. C. Harrison for calling our attention to the fact that Abramson [1940] had blanched large skin areas by adrenaline electrophoresis. The appearance of the blanched forearm may be seen in Pl. 1, figs. 3 *a, b.* The most intense blanching was obtained on cool skin; it lasted longer if the skin was subsequently exposed to air than if immersed in water. The technique for recording forearm blood flow was therefore adapted for use on the cool forearm, exposed to air. The blood flows in the cool limbs were physiologically subnormal; they differ from the previous ones in this important point.

Three experiments were done. The technique is illustrated by the following typical protocol:

Subject O. G. E. Recumbent on couch throughout experiment. Pneumatic cuffs on upper arms. Upper and lower rubber diaphragms for plethysmographs [Barcroft & Edholm, 1943] cemented to both forearms. Plethysmographs fitted later.

Time in min.

0–15	Rest. Forearms under blanket.
15–20	Nicely fitting pads and electrodes bandaged round both forearms between rubber diaphragms.
	Right pad soaked in saline at room temperature. Left pad soaked in 1/1000 B.D.H. Adrenalina (adrenaline base) adjusted to *p*H 4·5 with H_3PO_4 [Abramson, 1940] at room temperature.
20–33	Electrophoresis from 40 V. battery across 600 ohm resistance from which current was tapped to electrodes by slider (left electrode +). Increased to 10 mA. in first minute and decreased to zero during last minute.
33–35	Pads removed. 95% of underlying skin on test side intensely blanched with marked gooseflesh.
35–40	Infiltration of radial, median and ulnar nerves.
40–45	Plethysmographs slipped over each forearm and plated to rubber diaphragms. Pneumatic cuffs put on both wrists and inflated to 200 mm. Hg (till 60th minute) to arrest circulation in hands.
45–53	Simultaneous records of right and left forearm flow at 2 min. intervals (five sets for averaging).
53–55	Force of hand grip on test side reduced to a few grams; other movements paralysed.
55–60	Calibration of plethysmographs and recorders.
60–61	Wrist cuffs and plethysmographs taken off. Appearance of skin on test side unchanged.
61–64	Circulation in both forearms arrested for 5 min. On release the skin between the diaphragms on the control side flushed normally; on the test side no alteration in the colour of the intensely blanched skin took place.
72–74	Test forearm felt cooler than control. Skin temperature, taken thermoelectrically [Lewis, 1924], was: control 29·1° C.; test 26·9° C.
90	Slight adrenaline tremors.
210	Movement in test forearm returning. Action of anaesthetic prolonged, probably by cooling.

Great care was taken to expose both limbs to the same temperature so as to obtain comparable blood flows. During the preliminary rest both were covered by a blanket; during electrophoresis

the saline and adrenaline solutions were at the same temperature; after the nerve blocks the circulation in both hands was arrested to prevent warming of the test forearm by a rapid stream of blood from the vasodilated hand; during the recording of the flows both forearms were exposed to air in the plethysmographs. The conditions for cooling were the same on both sides; though the flows would be subnormal a difference would still indicate relative vasodilation on one side.

The evidence that the blood flow through the skin was smaller on the test than on the normal side while the flows were being recorded was as follows. After the flows were measured:

(1) 95% of the skin on the test side was still intensely blanched, and showed gooseflesh.

(2) Reactive hyperaemia could not be provoked in it.

(3) It was cooler than that on the control side.

Wayne [1933] electrophoresed adrenaline into small skin areas and found flushing could not be provoked by the introduction of small amounts of histamine or by procedures calculated to produce small amounts of 'H-substance intradermally. These facts and the intense blanching suggest that adrenaline introduced in this way arrests the circulation in the minute vessels. (In our experiments it would have been wrong to have described the blanched skin as bloodless; after firm digital pressure a return of colour was just perceptible.)

We found that when sweating was provoked in normal skin surrounding a blanched area the blanched skin failed to sweat. This is shown in Pl. 1, Fig. 4. Langley & Uyeno [1922] injected adrenaline subcutaneously into the cat's paw, and, under certain conditions, observed inhibition of sweating; when this occurred he attributed it to vasoconstriction of the afferent vessels of the glands; contraction of the plain muscle in the glands was not the factor. This probably signified that the inhibition of sweating we observed in human skin was the result of strong vasoconstriction in the depths of the skin.

Grant & Bland [1929] could find no arterio-venous anastomoses on the flexor surface of the forearm; the extensor surface was not examined. Grant [1929] found that, in the rabbit, arterio-venous anastomoses were constricted by adrenaline.

The above considerations suggest that the circulation in blanched skin was arrested.

Table 3 (I) and Fig. 1 (IV) show the results of three of these experiments. The average blood flow on the control side was 1·4 c.c., somewhat lower than normal owing to cooling. On the test side, with the skin circulation suppressed and the deep nerves blocked, it was 5·0 c.c., an increase of 3·6 c.c. or 257%.

TABLE 3. Blood flow in c.c./100 c.c. forearm/min. in the test, T, and control, C, forearm.
Both forearms cooling at the same rate

	Condition of test forearm	Subject	Test (T)	Control (C)	Difference $(T-C)$	% difference $\frac{100\,(T-C)}{C}$
I	Skin blanched Deep nerves blocked	A. S. E.	8·5	2·9	+5·6	+193
		Q. H. G.	4·5	0·9	+3·6	+400
		O. G. E.	2·1	0·4	+1·7	+425
		Average	5·0	1·4	+3·6	+257
II	Skin blanched	Q. H. G.	1·3	2·6	−1·3	− 50
		W. B.	1·0	1·8	−0·8	− 44
		O. G. E.	0·5	0·7	−0·2	− 20
		Average	0·9	1·7	−0·8	− 47

This increase might have been due to muscular vasodilation caused by deeply penetrating adrenaline. This was put out of court by the fact that suppressing

the circulation on the test side without blocking the deep nerves decreased the flow by 0·8 c.c. or 47 %, as shown in Table 3 (II) and Fig. 1 (V). The effect of cutaneous constriction had outweighed any muscular dilation.

The experiments therefore indicate that paralysis of the deep nerves increased the blood flow in structures beneath the forearm skin.

Conclusion

Blocking the radial, median and ulnar nerves approximately doubled forearm blood flow (Results and Control (I)). The effect was not caused by the adrenaline in the local anaesthetic (Control 4). In some experiments part of the sympathetic to the forearm skin was accidentally blocked. The hyperaemia caused by the release of tone in the cutaneous vessels was insignificant (Control 2). Therefore it was paralysis of fibres in the deep nerves that doubled forearm blood flow. Two possibilities were considered: increased forearm flow because motor nerve paralysis had abolished tonic skeletal muscular contraction and diminished resistance to the blood flow through muscle; increased forearm flow because sympathetic paralysis had released constrictor tone. The former was excluded on the grounds that in sympathectomized subjects deep nerve block had no effect on forearm blood flow (Control 5). Nerve block doubled forearm flow because it released constrictor tone. The loss of tone took place in vessels somewhere below the forearm skin. Dilation of the arterial tree may have increased the skin flow passively and accounted for some of the increased forearm flow. But when the circulation through the skin was suppressed deep nerve block still increased forearm flow (Control 5). The deep structures of the forearm are the muscle and a relatively small amount of bone. It therefore seems very probable that the vasodilation was mainly due to the paralysis of vasoconstrictor fibres to the forearm muscle. In so far as the possession of vasoconstrictor tone is concerned human muscle probably resembles dog [Anrep *et al.* 1934] and cat [Baetjer, 1930] muscle.

The probable significance of the vasoconstrictor tone in human muscle may be seen from the following. The average increase in forearm flow after blocking the deep nerves was 3·4 c.c./100 c.c. forearm/min., Table 1 (I). 100 c.c. of forearm contain approximately 1/450th of the body musculature (58·6% of the forearm volume is muscle [Abramson & Ferris, 1940]; 1·06 specific gravity of cat's muscle [*Tabul. biol., Berl.* 1925]; 40% of body weight muscle [Bardeleben, 1912]; body weight 70 kg.). Therefore releasing the sympathetic tone throughout the body musculature would probably increase its blood flow by something approaching 3·4 × 450 = 1530 c.c., say 1½ l./min.

Discussion

The conclusion that the sympathetic imposes tone on the vascular system in muscle seems opposed to that reached by Woollard & Phillips [1932], Friedlander *et al.* [1938] and Grant & Holling [1938]. The fact that blocking motor nerves did not raise muscle or overlying skin temperature seemed to gainsay its existence. The explanation may be as follows. Grant & Pearson [1938] found, and we have confirmed, that the skin and underlying muscle of an uncovered limb cool. This would reduce the vasodilation produced by the nerve block. For example, when the forearm was in water at 35° C. deep nerve block increased the flow by about 3·5 c.c./min. (Table 1 (I)); but in Prinzmetal & Wilson's [1936] experiments, at 24° C., blocking all the nerves to the forearm increased the flow by about 0·5 c.c. It seems, therefore, that the increase in blood flow caused by blocking motor nerves in a cooling limb might be too small to do more than retard the rate of cooling.

It is noteworthy that the release of sympathetic tone in muscle blood vessels could not explain the increase in the blood flow through them during exercise. Releasing sympathetic tone in the body musculature would probably increase its blood flow by about $1\frac{1}{2}$ l., but in severe exercise the increase is probably nearer 20 l. This accords with the established teaching that the hyperaemia in active muscle is brought about by the action of metabolites.

The existence of sympathetic vasoconstrictor tone in muscle does not imply that section of the nerve fibres concerned would be followed by permanent vasodilation. Grant & Holling [1938] found that the forearm was only hyperaemic for about a week after it was sympathectomized.

Summary

1. Blocking the nerves to the musculature of the forearm increases forearm blood flow (Lewis & Grant's plethysmographic method).

2. It does not do so in the sympathectomized forearm.

3. It does so in the normal forearm after the circulation in the skin has been suppressed.

4. These findings are discussed; the inference is that the vessels in human skeletal muscle probably receive sympathetic vasoconstrictor fibres and possess vasoconstrictor tone. In this respect they resemble those of the dog and cat.

5. The relaxation of sympathetic vasoconstrictor tone throughout the musculature of the body would probably increase its blood flow by something approaching $1\frac{1}{2}$ litres a minute.

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EXPLANATION OF PLATE 1

Figs. 3a, b. Show the intense constriction of the minute vessels of a large area of skin produced by adrenaline introduced by electrophoresis. The forearm skin was blanched as described in the text. The circulation through the arm was then arrested for a few minutes. The photographs were taken a short time after the circulation had been released, when conspicuous reactive hyperaemia had developed around the blanched area. The blanched skin did not redden. The shade of the blanched skin should be compared with that of the background, a white piece of cardboard. Gooseflesh is seen in Fig. 3b.

Fig. 4. Shows the inhibition of sweating of a large area of skin produced by adrenaline introduced by electrophoresis. To induce sweating both feet were immersed in hot water. Before sweating began the skin over the middle of the forearm was blanched. The skin of the forearm and hand was then treated by Minor's [1929] method so that sweating would turn it from light brown to black. In due course blackening occurred above and below but not in the electrophoresed skin. The photograph shows the blackening of the normal skin and the lighter colour of the electrophoresed skin proximal to it.

HISTAMINE IN NERVOUS TISSUE

By H. KWIATKOWSKI, *From the Clinical Research Unit, Guy's Hospital*

During recent studies on histamine metabolism and its relation to traumatic shock I found that extracts obtained from mixed somatic nerves contained relatively large amounts of histamine. This suggested the possibility of 'histaminergic' fibres in peripheral nerves, since the adrenergic and cholinergic nerves have a high content of acetylcholine and adrenaline respectively. Early observations by Osborne & Vincent [1900] suggest the presence of histamine in nerves but these have not been generally accepted [Halliburton, 1901].

Further observations showed that the histamine content of different nerves varies greatly, that on the whole the highest content is found in nerves responsible for antidromic vasodilation, and that histamine can be detected in venous blood after stimulation of the posterior roots. Owing to difficulties due to the war the observations are as yet incomplete, but they seem of sufficient interest to warrant publication now.

METHODS

Histamine content of nervous tissue. Histamine was extracted from various nerves in a number of species of animals (cats, rabbits, dogs, guinea-pigs, rats, monkey and man). Cats and dogs were killed with ether, rats and guinea-pigs by a blow on the head, and the monkey with chloroform. Human nerves were obtained within 12–48 hr. of death from patients dying of various diseases and air-raid casualties, the bodies being preserved in the refrigerator. Peripheral nerves, ganglia, spinal cord and brain tissues were dissected, weighed and washed with distilled water; the weight of the nerves varied between 20 and 200 mg. according to the size of the dissected nerve. Pieces of brain, cerebellum and spinal cord usually weighed from 500 to 2000 mg. Nervous tissue was placed in 10 c.c. of 10% trichloroacetic acid, minced and extracted for histamine by the method of Barsoum and Gaddum, modified by omitting the ether extract. The last stage of alcoholic extraction was retained because it removes potassium, which contracts smooth muscle but is insoluble in alcohol [Kwiatkowski, 1941].

The tissue extracts were assayed on the suspended guinea-pig's ileum perfused with Tyrode's solution containing half the normal $CaCl_2$ and equilibrated with 5% CO_2 in O_2 [Kwiatkowski, 1941].

The biological assay on the perfused gut has the advantages that it is usually very sensitive, allowing the use of small quantities of histamine, that it gives equal responses to similar doses and that only 0·1 c.c. of test fluid is needed. I have found, however, that sometimes, and most frequently in spring, the gut is relatively insensitive to histamine. It may be rendered more sensitive by first giving a few repeated doses of a large concentration of histamine, but the increased sensitivity may pass off after 15–30 min. It is known that different substances producing smooth muscle contraction, if given in different concentrations, do not show a similar gradient of contraction. In several experiments I have therefore compared the contractions produced by different concentrations of my extracts, the concentrations being a half, a fifth or a tenth, with similarly different concentrations of histamine solutions. I shall refer to this test as the dilution test.

The tissue extracts (cat and human nerves) were also tested in several cases on the blood pressure of atropinized cats under chloralose anaesthesia.

Antidromic stimulation of posterior roots and assay of histamine in venous blood and perfusate. Cats were anaesthetized with chloralose or dial. The peripheral ends of the cut nerves (L. V–VII) were stimulated faradically for a period of 1 min. (Palmer induction coil with 2 or 4 V. in the primary circuit, coil distance of 16–12 cm.). Care was taken that no muscle contractions occurred in the stimulated leg, since histamine may be liberated from a contracting muscle [Anrep, Barsoum, Talaat & Wieninger, 1939].

Samples of 1 c.c. of blood were withdrawn from the femoral vein shortly before, during and every minute after stimulation, and extracted. In a few experiments the plasma alone was used for extraction. In this case two samples of 5 c.c. of blood were withdrawn with a syringe containing 0·1 c.c. of 'Liquemin' (Roche) as anticoagulant. One sample was withdrawn before and the second during and after stimulation of the roots.

Previous experiments indicated that chloralose may produce a transient increase of blood histamine shortly after its administration. The chloralose was therefore given and the dissection made several hours before the experiment was performed, the animal meanwhile being kept warm.

Results

(i) *Histamine content of nervous tissue*

The first experiments carried out on the cat's nerves showed that the extracts contain a substance pharmacologically indistinguishable from histamine. The extracts caused a contraction of the atropinized ileum of the guinea-pig and,

if the dilution test was applied, there was no difference between histamine base and the active principle of the extracts. The extracts also caused a fall of arterial blood pressure in the atropinized cat. When the extracts were assayed against histamine the results obtained with both methods agreed quantitatively with each other. The active principle in cat's sciatic and brachial nerves was destroyed by ashing. In later experiments with human sciatic and vagus nerves, extracts were inactivated by incubation with histaminase (Torantil, Bayer). These facts make it likely that the active principle is histamine itself. I therefore express the activity of the nerve extracts in terms of histamine base.

The active substance is not formed during extraction with trichloroacetic acid since it is partially obtained by saline extraction. If nerves are chopped up in saline and left overnight in the ice-chest, the filtrate contains about half of the normal histamine yield of the nerve, and the other half can be extracted from the residue.

Somatic nerves. Table 1 shows the histamine equivalent per gram of fresh tissue of the brachial plexus and sciatic nerve in different animals. The highest values are obtained in rats and the lowest in rabbits. The histamine equivalent

TABLE 1. Histamine equivalent (μg./g.) of brachial plexus and sciatic nerve

	Brachial plexus	Sciatic nerve		Brachial plexus	Sciatic nerve
Cat	2·3	1·9	Monkey (*Macacus mulatta*)	1·4	2·2
	1·7	2·8	Man	1·3	3·0
	1·2	1·2		1·5	3·1
	0·56	3·0		1·7	
	2·5	1·5	Rat	3·0	3·4
		0·9		6·5	7·5
		2·25			11·0
		3·0			13·0
		1·1			6·2
Dog	2·3	2·5	Rabbit	0·3	0·3
	2·1	2·4		0·6	0·95
Guinea-pig	2·3	3·3			0·6
	1·4	1·6			
	4·0	4·0			
	5·0	5·0			

of these nerves appears to be independent of the presence of autonomic fibres. In two cats the left lumbar sympathetic chain was removed aseptically, and 5 and 7 days later the sciatic nerves were extracted. The left sciatic of the cat, operated on 5 days previously, contained 1·3 μg./g. histamine as compared with 1·5 μg./g. of the right side. In the other cat both sciatic nerves contained 0·9 μg./g.

Examination of a wider range of nerves, choosing those consisting mainly of motor or sensory fibres, reveals that the histamine is not distributed uniformly throughout the length of a nerve, and is not confined to either sensory or motor fibres. In general, the extracts of nerves consisting mainly of motor

TABLE 2. Histamine equivalent of nerves containing little histamine

Nerves containing mainly motor fibres	1	Oculomotor: Man 0·6; 0·4; 2·8; 0·8
	2	Trochlear: Man 0*; 0·2; 0·5
	3	Abducens: Man 0·6; 0·6; 0·1; 0·1
	4	Spinal accessory: Man 0; 0; 0·6; 0·07
	5	Hypoglossal: Man 0; 0; 0·6; 0·07
	6	Anterior roots: Cat 0; 0
		Dog 0
		Rabbit 0; 0
	7	Motor nerve to gastrocnemius: Man 0·5; 0·2; 0·6; 0·9; 0·2
		Cat 0·34; 1·8; 1·2
Nerves containing mainly sensory fibres	1	Lingual division of trigeminal nerve: Man 0·6; 0·6; 2·2; 0·3
		Cat 0·36
	2	Posterior roots: Man 0 (cervical region)
		Cat 0 (lumbar region)
		0; 0 (lumbar region)
		Dog 0 (lumbar region)
		Rabbit 0 (lumbar region)
		0 (lumbar region)
Nerves to special sense organs	1	Olfactory tract: Man 0; 0; 0; 0·8
		Monkey 0·2
		Cat 0; 0; 0
		Dog 0
		Rabbit 0
	2	Olfactory bulb: Man 1·0; 0·5; 0·3
		Cat <0·5
		Dog 0·4; 0·3
	3	Optic nerve: Man 0; 0; 0; 0·015; 0
		Monkey <0·2
		Cat 0; 0; 0; 0·03
		Rabbit 0; 0·04; 0·05

* 0 = <0·1 µg./g.

fibres and of sensory nerves from special sense organs (Table 2) have little or no action on the perfused gut. High histamine equivalents are obtained from distal parts of sensory nerves coming from the skin (Table 3). The histamine

TABLE 3. Histamine equivalent (µg./g.) of nerves containing
a high or medium content of histamine

Nerves containing mainly sensory fibres from the skin	Lateral sural cutaneous: Man 8·0; 6·4; 11·0; 7·0; 5·4
	Cat 3·2; 7·5; 11·0
	Rabbit 1·4
	Auricularis magnus: Rabbit 3·0; 1·5; 6·5
	Auricularis minus: Rabbit 2·15; 3·15; 3·3
	Ophthalmic division of trigeminal nerve: Man 1·8; 1·65; 6·0; 1·75
	Cat 2·0
	Maxillary division of trigeminal nerve: Man 3·0; 1·35; 4·2; 1·0
	Cat 1·6
Nerves containing mainly motor fibres	Posterior tibial: Man 4·3; 4·0; 1·5; 3·7; 4·2; 3·2
	Cat 2·0; 2·4; 2·7

content of posterior roots and of sensory roots of the trigeminal nerve must be very low, if any, and only a small amount can be detected in the lingual division. The semilunar ganglion yields a relatively high histamine equivalent, although this is lower than those obtained from the oph-

TABLE 4. Histamine equivalent (µg./g.) of trigeminal nerve

	Sensory root	Semilunar ganglion	Ophthalmic + maxillary divisions
Man	0*	0·45	2·5
	0	1·8	3·0
	0	0·85	1·3
Cat	0*	2·0	3·1
	0	—	1·75
	0	—	1·2
	0·07	—	1·6
	0	—	2·0
Dog	0*	1·1	2·0

* 0 = <0·1 µg./g.

thalmic and maxillary divisions distal to the ganglia (see Table 4). Fig. 1 illustrates the absence of a depressor action with the injection at I of the extract from the sensory roots (equivalent to 200 mg.), whereas injection at II of the extract from the nerves distal to the ganglion (200 mg.) has a depressor effect which was equal to 1 µg. of histamine (III).
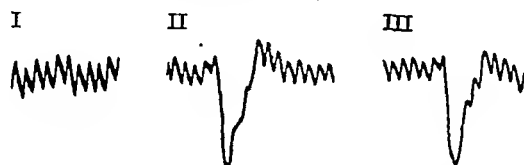
Fig. 1. Effects of extracts of trigeminal nerve of man on atropinized cat's blood pressure. I, 200 mg. sensory root; II, 200 mg. ophthalmic and maxillary division; III, 1 µg. histamine.

Among those nerves which supply chiefly skeletal muscles, the tibialis posterior only yields a relatively high histamine equivalent (Tables 2 and 3).

Degenerating sciatic nerve. Rats and cats were used. The left sciatic nerve was cut aseptically 3–105 days before the nerves were removed and extracted, the nerves of the right side being used as controls. The nerves of rats were pooled. The results are tabulated in Table 5. There was, in all experiments but

TABLE 5. Histamine equivalent (µg./g.) of degenerating sciatic nerve

Animal	Killed after section of left sciatic nerve days	Histamine in µg./g.		% increase in left nerve
		Right	Left	
2 rats	3	12·0	13·5	12·5
2 rats	15	11·0	7·1	− 35·4
2 rats	26	12·0	18·0	50·0
3 rats	35	11·0	18·2	65·4
3 rats	56	13·0	27·0	108·0
3 rats	68	6·2	40·0	545·0
1 cat	3	3·0	4·9	63·3
1 cat	105	1·75	6·1	248·0

one, an increase in the histamine equivalent of the degenerating nerve. From the figures of column 4 it appears that the increase is progressive and dependent upon the time allowed for degeneration.

Automatic nerves. The results vary in different species (see Table 6). In the rabbit the cervical vagus as well as the sympathetic has a low histamine content. In the one monkey at my disposal a low histamine content of the vagus

TABLE 6. Histamine equivalent (μg./g.) of autonomic nerves and ganglia

	Cervical vagus	Cervical sympathetic	Splanchnic (major)	Thoracic sympathetic chain including ganglia	Lumbar sympathetic
Cat	2·1	—	0·24	1·8	1·2
	2·3	—	—	1·8	1·4
	3·0	—	—	—	1·0
	2·2	—	—	—	—
	*4·5	1·6	—	—	—
Dog	4·5	—	—	—	1·25
Guinea-pig	6·7	—	—	—	—
Monkey	0 <0·3	—	—	—	1·4
Man	2·3	—	—	—	—
	2·25	—	—	—	—
	2·4	—	—	—	—
Rabbit	0·7	0 <0·2	—	—	<0·2
	—	0 <0·05	0	—	—

* = Without cervical sympathetic.

was found; the lumbar sympathetic had relatively more histamine. The vagus is rich in histamine in cats, dogs, guinea-pigs and man. Sympathetic nerves contain histamine although in smaller amounts. I have made no experiments to determine if the histamine is contained in the sensory or in the autonomic fibres of these nerves.

Central nervous system. Table 7 summarizes the results. Spinal cord and medulla oblongata contain barely measurable quantities of histamine. Brain

TABLE 7. Histamine equivalent (μg./g.) of central nervous system

	Spinal cord (lumbar region)	Medulla oblongata	Brain		Cerebellum
			Cortex	Mid-brain	
Cat	0 <0·1	0 <0·1	0 <0·1	0·28	2·0
	0 <0·025	0·0125	0·009	—	1·5
	0 <0·1	0 <0·1	0·12	—	—
	0·075	—	<0·2	—	—
Dog	0 <0·2	—	0·16	—	—
	0 <0·15	—	0·1	—	1·5
	0·2	—	—	—	—
Guinea-pig	0 <0·2	—	0·05	—	—
	0 <0·2	—	—	—	—
Monkey	0 <0·1	—	—	—	—
Man	0 <0·1	*	—	—	—
	0·08	—	—	—	—
	0 <0·2	—	—	—	—
Rabbit	0 <0·05	—	0·044	—	—
	0 <0·1	—	0·05	—	—
	0·03	—	—	—	—

* The extract showed the presence of a slow contracting substance. The tissue was obtained from a patient who died from uraemia.

cortex (parietal lobe and frontal lobe) contains only a small amount, 0·12μg./g. or less. The mid-brain of cat yields 0·28μg./g. and cerebellum 2·0 and 1·5μg./g. on extraction.

(ii) *Release of a histamine-like substance during and after stimulation of the posterior roots in cats*

Extracts of blood collected from the femoral vein in the absence of nerve stimulation cause, on the atropinized gut, only a small contraction, the effect of 1 c.c. blood being less than that caused by 0·01μg. histamine. Extracts of
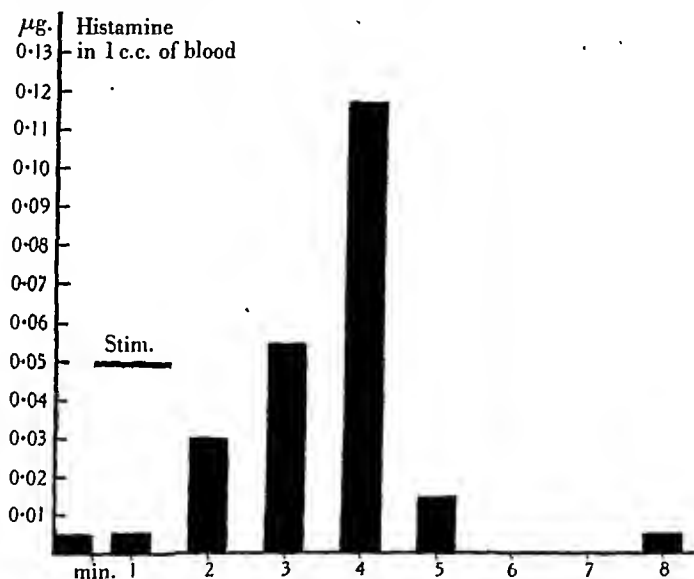
Fig. 2. Cat 2·3 kg. under chloralose anaesthesia. Stimulation of the cut peripheral ends of lumbar posterior roots (L. V–VII) for 1 min. with a coil distance of 14 om. The black columns reproduce the histamine equivalent of venous blood per o.c. in each minute sample.

blood collected during stimulation of the roots show usually no increased activity, but the extracts of blood collected 1–4 min. after stimulation produce a much stronger contraction of the atropinized gut. These results were obtained in six out of eight experiments and the histamine equivalents ranged from 0·03 to 0·18μg./c.c.

One experiment is illustrated in Fig. 2, which shows the histamine equivalents of the different samples of blood. The concentration of the active substance in the blood extracts is too low to be detectable, as histamine, on the arterial blood pressure of a cat. I have, however, been able to show that, like histamine, the active substance is insoluble in ether. The histamine-like substance is at least partly present in the plasma fraction of the blood. Control plasma samples in two experiments show activity less than 0·01μg./c.c. hista-

mine, while the samples obtained during and after nerve stimulation contain 0·03 and 0·025 µg./g. histamine respectively. It was not possible to determine whether nerve stimulation produced antidromic vasodilatation in all cases, because I was unable to obtain cats with unpigmented paws. In three of the cats, in which parts of the pads were unpigmented, definite flushing was seen to follow nerve stimulation.

In one experiment,.the hind limb of a cat was perfused through the femoral artery with Tyrode solution at 37° C., containing half its normal $CaCl_2$ content and equilibrated with O_2 and 5% CO_2. The inflow and outflow were kept constant throughout the experiment by using a perfusion pressure of 200 cm. of
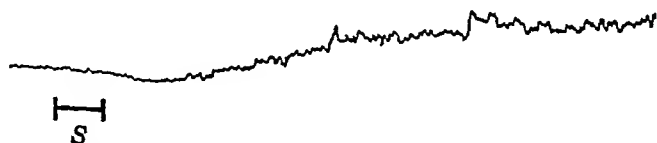
Fig. 3. Atropinized guinea-pig's gut perfused with perfusate from a cat's hind limb. At S the posterior roots (L. V–VII) were stimulated for 1 min. with a coil distance of 12·5 cm.

H_2O and inserting a capillary tube between the reservoir and the arterial cannula. The venous effluent was allowed to drop on the gut preparation at the rate of 10 drops in 6·5 sec. In the absence of stimulation no contraction of the gut occurred, the lever writing a horizontal line. Stimulation of the posterior roots produced a gradual increase in tone and spontaneous activity after a latency of about 1½ min. (see Fig. 3). The result was obtained repeatedly with renewed stimulation.

DISCUSSION

Lewis & Marvin [1927] brought forward strong evidence that stimulation of the posterior roots of the spinal cord liberates in the skin a dilator substance which is responsible for antidromic vasodilatation. According to them, the substance is histamine-like (H-substance), but they did not identify it as histamine. Attempts to identify the substance have given conflicting evidence. Kibjakow [1931] obtained a dilator substance in the venous effluent of the hind limb of the cat during and after stimulation of the lumbar posterior roots. This substance could be neither histamine, since it dilated the vessels of the perfused rabbit's ear which are constricted by histamine, nor acetylcholine, since it was stable in blood. Ungar [1935] obtained in dogs during antidromic stimulation of the posterior roots an acid gastric secretion which he believed to be due to the slow release of small amounts of histamine, and he introduced the term 'histaminergic' nerves. Later he withdrew this conception and assumed that the released substance responsible for the acid secretion was adrenoxine [Ungar & Parrot, 1939]. Wybauw [1936, 1938] found a release of

acetylcholine during antidromic dilatation, but his results were not obtained regularly.

The experiments described above leave little doubt that stimulation of the posterior lumbar roots liberates a substance which resembles histamine and is certainly not acetylcholine. Acetylcholine may be released, in addition, in antidromic vasodilatation as a result of the axon reflex which, induced by the released histamine, would liberate acetylcholine at the arterioles; such a possibility has been suggested by Dale [1929].

All nerves which have been shown certainly to possess the property of antidromic vasodilatation, with the exception of the lingual nerve, have a high histamine content. Thus the cutaneous nerves in those animals and to those skin regions for which antidromic vasodilatation has been established contain much histamine. The tibialis posterior nerve, which is mainly a motor nerve, supplies muscle and skin in which antidromic vasodilatation can be elicited and has a high histamine content. The nerves supplying the outer ear of the rabbit contain much histamine, whereas the nerves supplying the limbs contain little. Feldberg [1926] has shown that strong antidromic vasodilatation occurs in the outer ear of the rabbit, and it is probable, from the results of Wybauw [1938] on cats, that in the limbs it is confined to the most distal parts of the skin and muscles. In this case, the proportion of fibres in the sciatic nerve responsible for antidromic vasodilatation would be extremely small, and, even if these fibres did contain much histamine, this would increase only slightly the low histamine equivalent obtained from the whole nerve. The lingual nerve has a low histamine content. Antidromic stimulation of this nerve is known to cause a vasodilatation, but it has been shown that it receives its dilator fibres from cholinergic fibres from the chorda tympani [Heidenhain, 1883; Machol & Schilf, 1928].

Guinea-pig and rat nerves contain much histamine. Attempts to produce antidromic vasodilatation in the guinea-pig have been unsuccessful [Bena, 1930] and histamine has no dilator activity on the vessels of this animal. It is unknown if antidromic vasodilatation occurs in rats, and the rat's vessels are relatively insensitive to histamine.

The histamine content of nerves and the liberation of a histamine-like substance after stimulation of the posterior roots suggest the existence of 'histaminergic' nerve fibres as well as cholinergic and adrenergic nerves, but it is at present not possible to state definitely whether a high histamine content indicates the 'histaminergic' nature of a nerve. Whatever the answer will be, there are certain differences between the histamine-containing nerves and cholinergic nerves. In cholinergic nerves a high acetylcholine content is found throughout the whole length of a neuron, whereas histamine is low, or absent, in the nerve fibres passing from the sensory ganglion cells to the central nervous system. In cholinergic nerves the acetylcholine disappears early on degeneration,

whereas I find an increase of histamine under similar conditions. There is a possibility that, if histamine is released after nerve stimulation and during antidromic dilatation, the histamine may be released not only from the nerve endings but also from the tissue cells. In that case no close similarity should be expected between cholinergic and histaminergic nerves.

SUMMARY

1. A substance pharmacologically indistinguishable from histamine can be extracted from various nerves. This substance is inactivated after incubation with histaminase.

2. High values of histamine are found in distal parts of the sensory nerves coming from the skin and those nerves which are known to produce antidromic vasodilatation.

3. Nerves consisting mainly of motor fibres, sensory nerves from special sense organs and the central nervous system contain little or no histamine.

4. Stimulation of the cut posterior roots to the hind limbs of a cat liberates a histamine-like substance into the venous blood.

5. It is suggested that 'histaminergic' nerves exist as well as cholinergic or adrenergic nerves.

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SEASONAL AND ANNUAL CHANGES IN THE CALCIUM METABOLISM OF MAN

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Variations in the capacity of an individual to absorb Ca have been recorded and commented upon by previous investigators. Those who have interested themselves in the development and mineral metabolism of children have drawn attention to their waves of growth and pointed out that these will almost certainly be accompanied by waves of Ca retention, and possibly of Ca absorption [Nicholls & Nimalasuriya, 1939]. On the pathological side, rickets, tetany and osteomalacia have often been noted to be at their worst in the late spring, and for some twenty years this seasonal incidence has been attributed to the paucity of sunlight during the winter months. This, it has been supposed, led to the formation of so little vitamin D in the skin that the clinical signs of deficiency made their appearance or became more obvious, and from what is known of the effects of vitamin D these would certainly have been accompanied if not initiated by a lowered absorption of Ca from the gut.

Some people, who have studied mineral metabolism by means of balance experiments, have noticed that the amounts of Ca (or of Fe) absorbed might vary considerably from one analytical period to the next [Ascham, 1930–1; Nicholls & Nimalasuriya, 1939; Hutchison, 1937]. These 'paper' variations have generally been attributed to real changes in the biological activity of the gut. A proportion of them, however, have certainly been due to the fact that the analytical periods were too short to allow the faecal matter of one to be properly separated from that of the next. Variations of this kind may occur, but their reality is still very much obscured by technical uncertainties.

In the course of some investigations, which were originally undertaken for another reason, large seasonal variations in the absorption of Ca have been observed in certain individuals. Changes from one year to another have also been detected. Both these belong to types hitherto undescribed, and an account of their discovery and nature forms the substance of this article.

The setting of the observations

Early in July 1940, eight persons began a study of the effect of brown bread on Ca absorption and the effect of fortifying bread with Ca and diets with vitamin D. The experiments went on almost without a break till the latter end of March 1941, and they were restarted, with some of the original party, in August 1941. The seasonal changes were first noticed in November 1940 when some of the subjects were found to be absorbing dietary Ca less freely than they had done in July. By the end of March 1941 it was clear that the Ca metabolism of three of the original subjects had changed very much since they had first come under observation. Of the others, two had apparently shown no change and one an uncertain one. No statement could be made about two, who had left the party in September to take up work in Palestine, for they had not been under observation for long enough, or about their substitutes, for the arrangements demanded by the main experiments prevented the necessary comparisons being made on them. It may be said, therefore, that the seasonal changes presently to be described have been observed in three out of a possible six people. When this type of work was restarted in August 1941, it was found that the Ca metabolism of the five subjects who had taken part in the experiments in 1940 had altered appreciably since the previous summer. These annual changes, which were all of the same type and in the same direction, were demonstrable in every subject for whom the necessary (1940) data were available.

Experimental methods

Minerals have been determined in the food and in the excreta, and throughout this paper the term 'absorption' should be taken to mean the amount in the food minus the amount in the faeces. The 'balances' may be calculated from the presented data but only the absorptions will be discussed. A full description of the way in which these experiments were carried out, and of the subjects who took part in them, have been published [McCance & Widdowson, 1942a, b]. It is only necessary to state here that flour, either white or brown, always contributed 40–50 % of each person's total calories, and that the rest of the diet was freely chosen, but very similar from one experiment to the next. Most of the figures given in this paper are the results of three-week experiments, so that short-term variations and errors in faecal collection have been minimized or ruled out. Some are based upon two-week experiments and a very few on experiments which only lasted for eight days. At each season the absorption and excretion have been studied both on white bread and on brown, so that the whole series of results may be regarded as having been obtained in duplicate.

THE SEASONAL CHANGE

This effect has been observed in one man, R.M., and in two women, E.W. and B.A. The last was not under observation for quite such a long time as the other two. R.M.'s results are shown graphically in Fig. 1 and E.W.'s in Table 1. The 'negative' absorptions are an expression of the fact that the faeces sometimes had more Ca in them than the corresponding food had had.
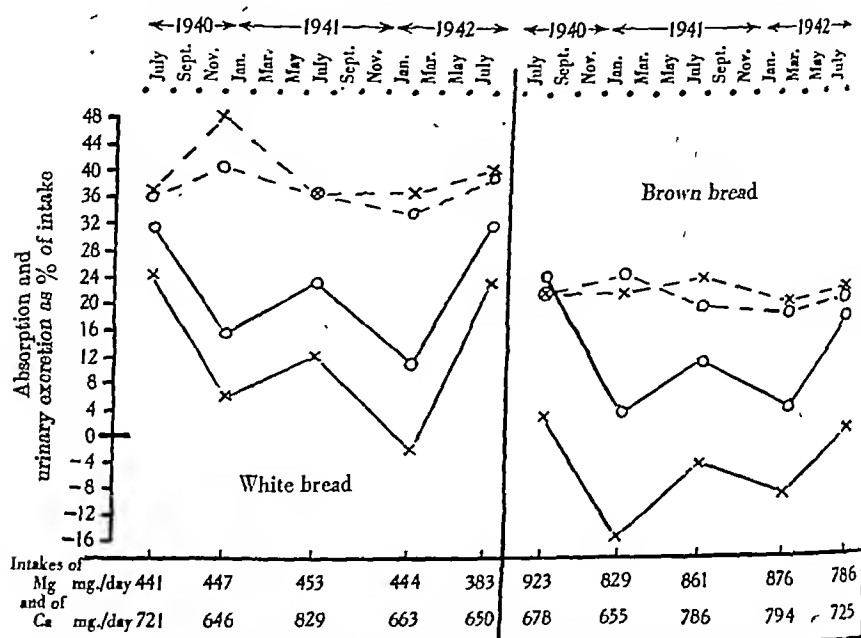
Fig. 1. R.M.'s absorption and urinary excretion of calcium and magnesium at different seasons from 1940 to 1942.

| × —— × | Ca absorption. | × – – × | Mg absorption. |
| ○ —— ○ | urinary Ca. | ○ – – ○ | urinary Mg. |

It will be noted that both subjects were under observation for three summers and two winters, and that throughout this time they both reacted to the seasons in precisely the same way. They absorbed Ca much more freely in July and August than they did in February and March, and the magnitudes of the differences were most striking. The seasonal effect was as clearly shown on brown bread as on white, but at any one season the absorptions were always lower on the former. Indeed, the results afford very pretty confirmation of the fact that brown bread inhibits the absorption of Ca [McCance & Widdowson, 1942 a, b]. It is interesting to point out in this connexion that the absorptions from the white bread diets became so bad in February 1942 that they were worse than the absorptions had been from the brown bread diets in August 1940.

The Mg absorptions were not affected by the seasons and showed a constancy om one experiment to the next which was really quite remarkable. R. M.'s bsorptions from brown bread diets illustrate this well. Although unaffected y the seasons, the Mg absorptions were of course affected by the degree to ʒhich the flour had been milled [McCance & Widdowson, 1942 *a*, *b*].

TABLE 1. The absorption and urinary excretion of calcium and magnesium by E.W. between 1940 and 1942

Type of diet	Date	Calcium			Magnesium		
		Intake mg./day	Absorption % of intake	Urine % of intake	Intake mg./day	Absorption % of intake	Urine % of intake
White bread	July 1940	450	26	27	299	39	35
	Dec. 1940 Jan. 1941 }	515	6	17	323	43	39
	July 1941	600	17	25	346	28	28
	Feb. 1942	535	7	15	323	44	37
	July 1942	590	29	30	305	41	39
Brown bread	Aug. 1940	550	9	18	720	22	20
	Feb. 1941	517	−6	8	552	20	22
	Aug. 1941	600	5	12	600	19	19
	Mar. 1942	625	0	9	635	21	19
	Aug. 1942	620	16	15	595	22	18

Table 1 and Fig. 1 also uphold the views which were put forward by McCance and Widdowson [1942 *c*], and which concern the relationship of the Ca and Mg excreted by the kidney to the amounts of these minerals absorbed from the gut. An increase in Ca absorption was regularly accompanied by a rise in the urinary excretion and vice versa. E. W. was frequently and R. M. invariably in negative Ca balance on these diets: both subjects were consistently in Mg balance, or nearly so.

THE ANNUAL CHANGES

If Fig. 1 and Table 1 are examined once more it will be seen that both R. M. and E. W. absorbed Ca better in the summers of 1940 and of 1942 than they did in that of 1941. This statement is true whether the comparison is made

TABLE 2. Annual changes in the absorption and excretion of calcium

Subject and year	White bread			Brown bread		
	Intake mg./day	Absorption % of intake	Urine % of intake	Intake mg./day	Absorption % of intake	Urine % of intake
E. B. 1940	500	61	53	530	32	34
1941	477	41	47	550	16	28
A. M. 1940	—	—	—	516	14	19
1941	—	—	—	556	1	11
B. A. 1940	381	32	34	497	9	24
1941	493	22	25	517	9	11

when white bread or brown formed the basis of the diets. Table 2 summarizes the data of three other persons whose metabolism was studied in 1940 and in 1941. It will be noted that they also absorbed Ca better in 1940 than they did

in 1941. B.A.'s results on brown bread should not be regarded as an exception. Her 1941 experiment was one of the very few which only lasted for 8 days, and consequently her absorption was less accurately measured than usual. Her urinary output was very much lower at that time than it had been on the same diet the year before. 1941 was evidently a worse year for Ca absorption than 1940, and R.M.'s and E.W.'s extension of these experiments suggests that 1942 was a better year than 1941 and as good as 1940. The Mg absorptions were equally good in 1940 and 1941 (see Fig. 1), and the Mg figures for E.B., B.A. and A.M. have not been given.

The absorption of phosphorus

The absorption and excretion of P have been followed in parallel with those of Ca and Mg, but no changes, either annual or seasonal, have been detected, and the figures have not been given.

DISCUSSION

Before discussing the cause and consequences of these variations in people's ability to absorb Ca, it is essential to establish the fact of their biological reality. People unfamiliar with the accuracy with which this sort of experiment can be carried out, or unaccustomed to the almost clock-like regularity of a single person's metabolism under standardized conditions, may be inclined to dismiss them as the product of chance irregularities in the subject's behaviour or even of technical errors. The authors are satisfied as to the biological reality of the variations for the following reasons: (1) Their magnitude. The differences between the absorptions of R.M. and E.W. in the summer of 1940 and the spring of 1941 were quite outside any possible error in faecal collection or method of analysis. The annual changes were not so large as the seasonal ones, but every subject showed them and they could not have been technical in origin. (2) The confirmatory variations in the urinary Ca [McCance & Widdowson, 1942 c], to which reference has already been made. (3) The constancy of the Mg absorptions and urinary excretions. These acted as a check on the collections and general technique and showed up the fluctuations in Ca metabolism by providing the contrast of their own stability. (4) The duplication of the whole set of results at two levels of Ca absorption. This was the object of continuing to study the absorptions on both white and brown bread, and, looking back, the extra work would seem to have been well repaid.

The causes of these variations must next be considered. When the seasonal effects were first observed, it was thought that they were probably due to changes in the amount of vitamin D in the food or in the amount formed by the sun in the skin. This was a natural hypothesis to adopt in the light of current ideas, and the one which Havard & Reay [1925] put forward to account for the seasonal changes in the inorganic P of the serum, first observed in

ildren by Hess. & Lundagen [1922]. Accordingly, when the brown bread xperiment of February 1941 was over, the diets were not relaxed and 2000 u. of vitamin D were administered daily to B.A., E.W. and R.M. for seven reliminary and twenty-one experimental days. The drug was given in the orm of calciferol dissolved in arachis oil [McCance & Widdowson, 1942 *a*]. The ffect of vitamin D on the Ca absorptions and excretions are shown in Table 3.

TABLE 3. Effect of vitamin D on the absorption and urinary excretion of calcium

	Control period Feb. 1941			Vitamin period March 1941		
Subject	Intake mg./day	Absorption % of intake	Urine % of intake	Intake mg./day	Absorption % of intake	Urine % of intake
B.A.	500	3	15	501	1	9
R.M.	655	− 15	4	700	−8	4
E.W.	517	− 6	8	522	8	9

Had D acted potently, as one might have expected it to do, the absorptions certainly, and also to some extent the urinary excretions, should have increased. As it was E.W.'s absorptions improved, R.M.'s also increased a little, but there were no appreciable changes in their urinary excretions. B.A.'s absorption fell very slightly and her urinary excretion more definitely. Since it is unlikely that these three adults were deriving so much as 2000 i.u. of D per day from their summer food or from the limited amounts of sunlight to which they were able to expose themselves in the warm weather, these findings made it impossible to maintain the view that a deficiency of vitamin D was the cause of the poor winter absorptions and low urinary excretions.

It was next supposed that the variations might not be seasonal in origin but that high cereal diets taken over a long period of time—eight or nine months in this instance—might depress the absorption of Ca. This has been excluded by the passage of time, for since March 1941 E.W. has eaten very little bread except during the metabolism experiments and R.M. practically none.

Any theory as to the origin of these changes must take account of the facts that: (1) All the five subjects absorbed Ca better in 1940 than they did in 1941, but only three of the five were affected by the passage from summer to winter. Hence one must suppose that the annual and seasonal fluctuations had different metabolic origins. (2) The Mg absorptions did not change. This is important, for it excludes non-specific changes in the membranes lining the alimentary canal. The only substance known at present which influences Ca but not Mg absorption is vitamin D, and although variations in the supply of this vitamin do not seem to have been responsible for the changes which have been observed, it is tempting to try to explain all of them in terms of vitamin D. This can be done if one supposes that they were caused by variations in the resistance or in the responsiveness of these adults to the vitamin D already in the body or taken with the food. This is a reasonable hypothesis. It covers all the present observations and fits the known facts about the Ca and vitamin D

metabolism of adults. Healthy men and women, who are not in need of D, are very resistant to it. This must be so, for otherwise it would not be necessary to give such enormous doses to make their Ca metabolisms alter in the characteristic way. Furthermore, children are known who respond very sluggishly to vitamin D, and there is a form of rickets which can only be cured by colossal doses. It has been shown that children with this disease absorb the vitamin [Bakwin, Bodansky & Schorr, 1940], so that in them tissue resistance is a proven fact, and one may compare it with the better known resistance to insulin, which may develop in certain diabetics. It is suggested, therefore, that both the annual and seasonal variations in the ability of these adults to absorb Ca were primarily and directly due to variations in their responsiveness to the vitamin D available to them, but that these variations in responsiveness were due to unknown and probably different metabolic origins. If an adult's responsiveness to vitamin D does alter from one time of year to another it might be possible to demonstrate that persons, whose Ca absorptions exhibited seasonal variations, were more easily affected by administered vitamin D in the summer than they were in the winter. If the response to D does vary from one year to another and from one season to another, why it does so becomes the problem, and at present there appear to be no clues at all. Seasonal changes in avian Ca metabolism are well known and might be helpful, but man is a much less seasonal animal and has a very different metabolism. Some progress might be made if these annual and seasonal phenomena were to be studied simultaneously by observers in Europe, North America, South Africa and Australia, but this would require considerable organization.

The significance of these observations seems to be twofold—practical and theoretical. From the purely practical point of view, all those interested in human Ca metabolism should be alive to the possibility of having their experiments vitiated or upset by fluctuating Ca absorptions. If the studies are to continue over a period of months, it may be necessary to run two or even more control experiments, and it is obvious that changes of the kind just described might greatly interfere with an attempt to study the effect of pregnancy on Ca metabolism. It must be remembered also that only certain persons can be expected to show seasonal instability, and at present there is no way of predicting whether any given individual is likely to do so or not. Furthermore, although 1941 was demonstrably a worse summer for Ca absorption than 1940 or 1942, it is impossible to give any estimate at present of the size of variation to be expected from any one year to the next. Theoretically these results are stimulating if not at present very constructive. The fluctuations in Ca absorption appear to be due to some factor or factors in Ca metabolism hitherto undescribed and which will ultimately have to be defined and characterized. If seasonal variations in a person's responsiveness to D should be substantiated by future findings, some similar phenomenon may very well turn out to under-

lie the more or less permanent differences which two perfectly normal people may exhibit in their ability to absorb Ca. The more one considers these personal differences, the more interesting they appear to be, and it is odd that they should have attracted so little attention. It is certain that the Mg absorptions are not subject to the same control—whatever it may be [McCance & Widdowson, 1942 *a*, *b*].

SUMMARY

1. Large seasonal variations in Ca absorption have been demonstrated in three out of six people who were taking part in a metabolism experiment. Least Ca was absorbed in February and March; most in July and August.

2. All persons—five in number—who were under observation in the successive summers and autumns of 1940 and 1941, absorbed Ca more freely in the former year.

3. All changes in absorption were accompanied by corresponding changes in urinary excretion.

4. The Mg absorptions and urinary excretions did not fluctuate in the same way and remained very constant over the whole period of investigation.

5. The administration of 2000 i.u. of vitamin D in March 1941 did not materially improve the absorption or urinary excretions of Ca.

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EFFECTS OF X-RAYS ON ACETYLCHOLINE SOLUTIONS SHOWING THE DILUTION AND PROTECTION PHENOMENA FOUND FOR ENZYMES

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In two previous papers [Dale, 1940, 1942] the effect of X-rays on enzyme solutions has been investigated quantitatively and, without repeating the details, it has been shown that a dilute enzyme solution can be inactivated by a given dose of X-rays more completely relative to its initial concentration than can a more concentrated one, and the implications of this phenomenon have been discussed. It has also been shown that organic non-enzymatic substances dissolved in the enzyme solution inhibit the radiation effect. From this inhibition phenomenon it could be assumed that the mode of action of the radiation was not confined to enzymes but could be extended to any organic substance. A direct proof, however, which is not based on enzymatic reactions as indicators of radiation effects, is still needed, and it is the purpose of this paper to show that the effect of X-rays, found to hold for enzyme solutions, can be extended to include non-protein, non-enzymatic substances.

Acetylcholine (denoted in what follows by ACh.), has a powerful depressing effect on the contraction of the heart muscle when used in concentrations comparable to that of enzymes. It was, therefore, hoped that the effect of X-rays on ACh. solutions could be demonstrated when Clark's technique of testing ACh. solutions on the isolated ventricular strip of the frog's heart was employed. I am indebted to Dr W. Schlapp for having drawn my attention to Clark's method as an alternative to the use of the muscle of the leech which is unobtainable at present.

Clark [1926] has given a detailed account of the relation between the action of ACh. and its concentration. In the first place the activity varies 100-fold for a 10,000-fold change of concentration of ACh., i.e. the reaction of the muscle to a change of concentration is relatively small, and therefore greater doses of X-rays compared with those used for enzymatic solutions will be required to show marked inactivation effects. Furthermore, the approximately linear drop in action obtaining for medium concentrations of ACh. flattens out for decreasing concentrations, so that roughly a 60-fold drop in concentration is

required to remove the last 20% of action as compared with a 5-fold drop to lower the action from 60 to 40%. The test, therefore, is more sensitive to a change of concentration in this steeper part of the curve, i.e. towards increasing concentration. On the other hand, as shown in the author's first paper, increasing concentration requires increasing the X-ray dose to obtain a given percentage destruction. The radiosensitivity of this biological test is, therefore, lower than that of enzymatic reactions.

METHODS

To show a sufficiently marked radiation effect the following layout and technique of the experiments were adopted. Two ACh. solutions, W and S, differing 10,000-fold in concentration, were exposed to an X-ray dose of 40,000 r. Solution W was $2 \cdot 5 \times 10^{-6}$ M and solution S was $2 \cdot 5 \times 10^{-2}$ M with respect to ACh. The stronger solution S was diluted 10,000 times after irradiation. In the tracings which follow, solution W after irradiation is marked W^* and solution S after irradiation and dilution SW^*. An unirradiated portion of solution W was kept as control. The ACh. content of the solutions W, SW^* and W^* was then tested on the ventricular strip of the heart.

The test of the activity of the ACh. solution on the heart muscle has to be carried out in Ringer's solution. During irradiation, however, the ACh. solution should not contain the Ringer constituents which must, therefore, be added after irradiation. For this purpose the NaCl, KCl and $CaCl_2$ of Ringer's solution were dissolved in 0·6 ml. of water in such a concentration that addition of 24 ml. of the irradiated solution made their concentration identical with that of Ringer's solution as used by Clark. Then 0·6 ml. of the remaining Ringer component $NaHCO_3$ was added in a likewise appropriate concentration.

This division of the Ringer components and their order of mixing is necessary to avoid precipitation of the calcium and the contact of ACh. with a too alkaline solution.

For the actual test of the ACh. solution Clark's technique was adopted without major alterations, though it should be mentioned that it was found advantageous to allow the heart muscle to settle after its preparation for about 2 hr. and then to stimulate it with regular break induction shocks for about 15 min. in Ringer's solution before the actual recording of the muscle's contraction in the experimental solutions was started. For each change of fluid the electrical stimulation was interrupted for about 8 sec. The frequency of the stimulation was usually 30 per min.

IRRADIATION

The ACh. solutions were irradiated with rays of half-value layer of 6 mm. Cu from a 500 kV. Metropolitan-Vickers continuously evacuated X-ray tube with a cool window. The dosage rate was approximately 370 r. per min.

The 'dilution' effect

A tracing of a typical experiment is shown in Fig. 1 A, B. In A as well as periods of immersion in Ringer's solution without ACh. were inserted between each change of A.Ch. solution, and in both tracings the irradiated weak solution (W^*) shows the least depressing effect on the muscle's contraction, indicating a reduced ACh. content, whereas the unirradiated control solution (W) and the irradiated strong solution diluted after irradiation (SW^*) indicate an identical ACh. content.

R   W*   R   W   R   SW*   R

Fig. 1A.

R   W   R   SW* · R   W*   R

Fig. 1B.

Fig. 1. A. The effect of irradiated and unirradiated solutions of acetylcholine on the frog's heart R = Ringer's solution; W = acetylcholine $2 \cdot 5 \times 10^{-6}$ M; W^* = acetylcholine $2 \cdot 5 \times 10^{-4}$ M irradiated; SW^* = acetylcholine $2 \cdot 5 \times 10^{-2}$ M irradiated and after irradiation diluted 10 times. B. As A, with reversed order of immersion.

Tracing B differs from A only in the order of immersion of the muscle into the various solutions, demonstrating that the results are independent of the order of immersion.

Fig. 2 shows the same reduced ACh. content in W^*, the difference of procedure being that the change from one fluid to the other was done directly

R   W   W*   SW*   R   W*   SW*   W   R

Fig. 2. Immersion without intervening Ringer periods; in the second half of the tracing order of immersion changed.

without intervening Ringer periods. The gradual increase of the muscle contraction for W^* shown in the first half of the tracing to its proper final value demonstrates that a change from a more concentrated solution to a weaker one has taken place. In the second part of the tracing after one Ringer period, the order of immersion was changed again. These tracings prove that the weak solution is inactivated more completely relatively to its initial concentration than the strong one.

The 'protection' effect

In a similar way, it can be shown that the presence of another organic substance in the ACh. solution during irradiation inhibits the radiation effect. Glucose, being innocuous to the working of the muscle, was chosen as an inhibitor.

For this experiment the following solutions were required:

For irradiation $\begin{cases} (X) & \text{ACh. } 10^{-6} \ M. \\ (Y) & \text{ACh. } 10^{-6} \ M \text{ containing glucose of the concentration of } \\ & \quad 1 \cdot 1 \times 10^{-2} \ M. \end{cases}$

     (Z) ACh. 10^{-6} M as an unirradiated control.

Solutions X (after irradiation) and Z as well as the Ringer's solution are made up to the same glucose content as solution Y.

Fig. 3. The effect of irradiated and unirradiated solutions of acetylcholine on the frog's heart in the presence and absence of glucose during irradiation, with intervening Ringer periods. R =Ringer's solution; A =acetylcholine 10^{-6} M; A^* =acetylcholine 10^{-6} M irradiated; $[A+G]^*$ =acetylcholine 10^{-6} M irradiated in the presence of $1 \cdot 1 \times 10^{-2}$ M glucose.

Fig. 4. The same as Fig. 3 without intervening Ringer periods.

The experiments have been carried out again with (Fig. 3) and without (Fig. 4) intervening Ringer periods. In both tracings the presence of glucose in the ACh. solution ($[A+G]^*$) protected against the irradiation effect, whereas the ACh. solution without glucose (A^*) was reduced in activity.

DISCUSSION

The results of the present paper cannot be seen in their right perspective without briefly recalling relevant points of the two previous papers on the enzymes carboxypeptidase, polyphenoloxidase and d-amino-acid oxidase. There the dilution and the protection phenomena in their relation to the inactivation of these enzymes have been shown. The results excluded the possibility of a direct action of radiation on these enzymes and could be understood only if the X-rays form an intermediate product from the water which in turn reacts with the enzymes. Since many organic, non-enzymatic, non-protein substances protected the enzymes against inactivation by X-rays, it was assumed that the mode of action of radiation could be extended to these substances. A fair

justification for this assumption was provided by the inactivation of the organic prosthetic group of the amino-acid oxidase—alloxazinadenine dinucleotide— itself non-enzymatic and non-protein, though still related to an enzyme.

The present ACh. experiments are not based on enzymatic reaction at all, and therefore show clearly that both the dilution and the protection phe- nomena hold for a representative of biologically active, organic, non-protein substances, and that such substances obey the same laws of inactivation by X-rays as do enzymes.

Another point of importance only deduced in the discussions in the two previous papers now appears to be demonstrated experimentally. There it was stated that radiosensitivity, in the biological sense, is not a fixed entity but a variable, the value of which depends on the concentration of the material sub- jected to radiation, on the presence of 'protecting' substances and, by deduc- tion, on its particular function within the living cell. The response produced by acetylcholine varies only narrowly for large variations of its concentration. Any absolute radiation effect therefore appears to be expressed on a diminished scale in terms of this response. This instance of radiation effect shows that in living material a reaction of this kind must be far less important from the point of view of radiosensitivity than that found for enzymes, for which radiation effects are expressed on a greatly enlarged scale in terms of the response measured, owing to the catalytic nature of their action.

SUMMARY

1. Solutions of acetylcholine as a representative of organic, non-protein, biological substances, entirely unrelated to enzymes, have been exposed to X-radiation and then tested for their biological activity on the isolated ventricular strip of the frog's heart stimulated by regular break induction shocks.

2. It is shown that a dilute solution is inactivated by a given dose of X-rays more completely relatively to its initial concentration than a more concentrated one, and that glucose present in the acetylcholine solution during irradiation inhibits the radiation effect.

3. This proves that the mode of action of X-rays on acetylcholine is the same as that previously found to hold for enzymatic solutions.

4. The significance of these results and the relative radiosensitivity of acetylcholine as compared with enzymatic reactions are discussed.

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THE OXYGEN AFFINITY OF HUMAN MATERNAL AND FOETAL HAEMOGLOBIN

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The present communication records a comparative investigation of the oxygen dissociation curves of human maternal and foetal haemoglobin. Previous work [McCarthy, 1933] has shown that foetal haemoglobin in the goat has a higher affinity for oxygen than the maternal haemoglobin. Similar results were obtained by Hall [1934] using dilute solutions of haemoglobin from the goat, sheep and fowl. Haselhorst & Stromberger [1931] studied human maternal and foetal blood by determining the relationship between the K of Hill's formula and the hydrogen-ion concentration, assuming that n is a constant. Oxygen dissociation curves determined by calculation differed in position, the foetal curve being found to the left of the maternal. Eastman, Geiling & de Lawder [1933] and Leibson, Likhnitzky & Sax [1936] published experimentally determined curves which substantiated the difference in position, in agreement with the findings of Barcroft, Elliot, Flexner, Hall, Herkel, McCarthy, McClurkin & Talaat [1934] on the goat and those of Roos & Romijn [1938] on the cow. Haurowitz [1935] and R. Hill & Wolvekamp [1936] have stated, however, that in dilute solution human foetal haemoglobin has a lower affinity for oxygen than maternal haemoglobin, and that this relationship is reversed when dilute suspensions of corpuscles are employed. Their results were obtained by spectroscopic methods, and it seemed desirable that such data should be compared with gasometric measurements made by standard methods on more concentrated haemoglobin solutions and corpuscle suspensions.

Preparation of haemoglobin solutions

Samples of blood were obtained from the umbilical cord at birth and from the mother by arm venepuncture within an hour of delivery. The corpuscles were washed with salt solution and the stromata were removed according to the method described by Adair & Adair [1934]. The preparations were dialysed

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in collodion membranes with distilled water and subsequently with Sorensen phosphate buffer mixture containing $\frac{1}{30}$ mol. of disodium hydrogen phosphate and $\frac{1}{30}$ mol. of potassium dihydrogen phosphate per litre of solution. The pH value of this mixture is about 6·78 at 38° C. The pH value of 6·8 was chosen in preference to the more physiological 7·4 for reasons discussed in a previous paper [McCarthy, 1933]. In the experiments numbered 11, 12, 13, 14 and 20 in Table 1, the haemoglobin solutions were dialysed against the isotonic buffer

TABLE 1

Exp. no.	Nature of preparation	Source	Buffer	Oxygen pressure in mm. Hg at half saturation	n	Oxygen capacity c.c./ 100 c.c.
1	Solution of haemoglobin	Normal	Dilute	26	2·18 ± 0·18	15·04
2	„	„	„	27	2·32 ± 0·13	17·80
3	„	„	„	23	1·8 ± 0·13	9·82
4	„	Maternal	„	27	2·2	4·28
	„	Foetal	„	28	1·7	15·76
5	„	Maternal	„	21	1·7 ± 0·20	12·17
	„	Foetal	„	26	1·9 ± 0·13	9·60
6	„	Maternal	„	25	2·1 ± 0·2	4·90
	„	Foetal	„	30	2·2	10·08
7	„	Maternal	„	24	2·25 ± 0·15	10·95
	„	Foetal	„	26	1·85 ± 0·15	13·20
8	„	„	„	24	1·5	11·90
9	„	„	„	19	1·7	7·34
10	„	„	„	21	2·13	5·39
11	„	Maternal	Concentrated	32	2·7 ± 0·2	7·85
	„	Foetal	„	38	2·5	18·33
12	„	Maternal	„	31	2·3 ± 0·2	9·15
	„	Foetal	„	38	2·6 ± 0·2	14·98
13	„	Maternal	„	33	2·82 ± 0·01	14·00
	„	Foetal	„	38	2·8 ± 0·0	15·41
14	„	Maternal	„	31	2·5	19·80
	„	Foetal	„	33	2·1	22·00
15	Suspension of corpuscles	Maternal	„	41	2·64	14·62
	„	Foetal	„	36	2·46 ± 0·12	16·60
16	„	Maternal	„	43	2·5	10·50
	„	Foetal	„	38	2·2 ± 0·1	20·14
17	„	Maternal	„	40	3·0 ± 0·0	10·72
	„	Foetal	„	37	2·86	14·37
18	„	Maternal	„	40	2·4	17·90
	„	Foetal	„	40	2·5	15·52
19	„	Maternal	„	42	—	6·60
20	„	Normal	„	40	—	16·83
	Solution of haemoglobin	„	„	31	2·8	16·00

described in the next paragraph, to ensure that the measurements on solutions and on corpuscles were made under comparable conditions as regards pH value and salt concentration.

Preparation of corpuscle suspensions

Samples of blood were centrifuged and the plasma was removed. The corpuscles were washed three times with isotonic phosphate buffer containing $M/14$ mol. of potassium dihydrogen phosphate and $M/14$ mol. of disodium hydrogen phosphate per litre of solution. The pH of this mixture at 38° C. is approximately 6·8. The corpuscles were then suspended in the buffer solution and stored at 0° C. for periods from 1 to 3 days.

TABLE 2

Exp. no.	Source								
1	Normal	Pressure in mm. Hg	24	26	29·5	41·6	42·7	63·2	—
		% saturation	45	47·5	59	75·6	76	84·3	—
2	,,	Pressure in mm. Hg	7	22	31·7	33	41	66·4	—
		% saturation	6·5	37·4	60	60·3	73·5	90·4	—
3	,,	Pressure in mm. Hg	18	32·3	48·2	52	59·6	—	—
		% saturation	41·5	66	80·7	83	84·6	—	—
4	Maternal	Pressure in mm. Hg	16·3	26·5	34·3	51	—	—	—
		% saturation	23·6	50·4	62·3	79·5	—,	—	—
	Foetal	Pressure in mm. Hg	21·4	26	32·3	42·2	59	62·2	80·2
		% saturation	34	47·7	57·7	69	79·4	79	86·3
6	Maternal	Pressure in mm. Hg	19·2	27·6	36·6	56·6	—	—	—
		% saturation	40	53	75	85	—	—	—
	Foetal	Pressure in mm. Hg	16	24	32·4	34·2	45·6	—	—
		% saturation	21·3	39·7	52	60	73·2	—	—
7	Maternal	Pressure in mm. Hg	16·8	29	44·5	—	—	—	—
		% saturation	31·4	56	82	—	—	—	—
	Foetal	Pressure in mm. Hg	17·2	26·8	42·7	52·8	—	—	—
		% saturation	35·3	48	72·3	78·6	—	—	—
8	,,	Pressure in mm. Hg	11·1	25·5	—	—	—	—	—
		% saturation	24·5	52·3	—	—	—	—	—
9	,,	Pressure in mm. Hg	9·03	20	20	—	—	—	—
		% saturation	21·6	52·4	53	—	—	—	—
10	,,	Pressure in mm. Hg	14·4	20·7	19	32·1	34·2	43·5	44·2
		% saturation	33·2	49·3	47	69	71	85·4	86
11	Maternal	Pressure in mm. Hg	20	38	42·5	56	—	—	—
		% saturation	21	65	72	80·7	—	—	—
	Foetal	Pressure in mm. Hg	29·5	39·3	48·8	55	—	—	—
		% saturation	35·5	53·2	65·8	75	—	—	—
13	Maternal	Pressure in mm. Hg	21·4	35·2	48	62·5	—	—	—
		% saturation	24·4	55	72	87	—	—.	—
	Foetal	Pressure in mm. Hg	20·5	37·4	45·5	51	65	65·2	—
		% saturation	21·2	50·7	60·6	74·7	85·7	88·4	—
18	Maternal	Pressure in mm. Hg	10·6	31	38·6	46·6	54	75·6	—
		% saturation	7·8	34	47	59	73·4	83	—
	Foetal	Pressure in mm. Hg	14·5	22·7	36	46	64	80	—
		% saturation	9·4	17·5	40·2	59·4	77	82·4	—
19	Maternal	Pressure in mm. Hg	15	21	26	54	—	—	—
		% saturation	7·8	16·3	20·4	74	—	—	—
20	Normal corpuscles	Pressure in mm. Hg	32·2	38	47	—	—	—	—
		% saturation	29·7	46	58·2	—	—	—	—
	Normal haemoglobin	Pressure in mm. Hg	15·6	20·6	30·7	36	44·3	57	—
		% saturation	16·2	18·2	47·5	64·2	73	84	—

Oxygen dissociation curves

The haemoglobin solutions and corpuscle suspensions were equilibrated at 37° C. in small saturators of 30–60 c.c. capacity described by Barcroft [1934]. For each point, one determination of the percentage oxygenation was made in 0·4 c.c. of fluid by the Van Slyke manometric method. The oxygen pressure was measured by duplicate Haldane analyses on the gas phase.
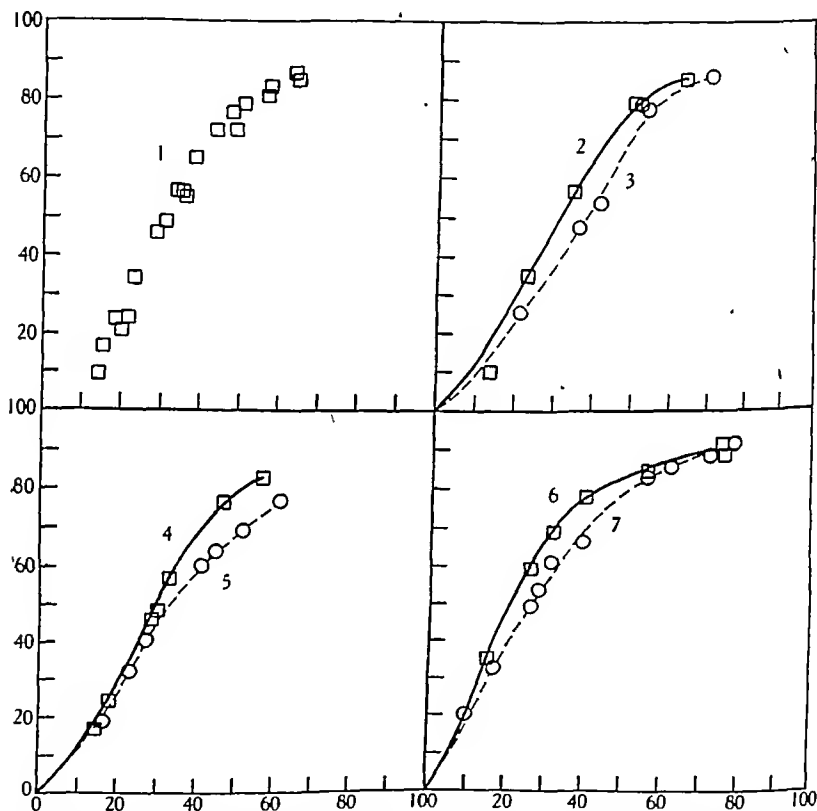
Fig. 1. Oxygen dissociation curves of haemoglobin in solution: circles, foetal; squares, maternal. Ordinates: percentage oxygen saturation. Abscissae: oxygen pressure in mm. Hg. 1, composite curve of maternal haemoglobins. Curves 2 and 3, Exp. 12. Curves 4 and 5, Exp. 14. Curves 6 and 7, Exp. 5 (buffer $M/15$). Oxygen capacities are recorded in Table 1.

Table 1 gives for each preparation the oxygen capacity, the value of n calculated by Hill's equation, and the oxygen pressure at which the haemoglobin is half oxygenated. The percentage saturation at varying pressures of oxygen for different solutions are recorded in Table 2.

Measurements on haemoglobin will be considered first. The curve numbered 1 in Fig. 1 is a composite curve of maternal haemoglobins dialysed against

isotonic ($M/7$ phosphate) buffer mixture. The small range of variation indicates that the component curves are probably identical.

The curves for foetal haemoglobin are to the right of the maternal, although in some cases the difference in position is very slight. The pair of curves numbered 2 and 3 from Exp. 12 and the curves 4 and 5 from Exp. 14 were
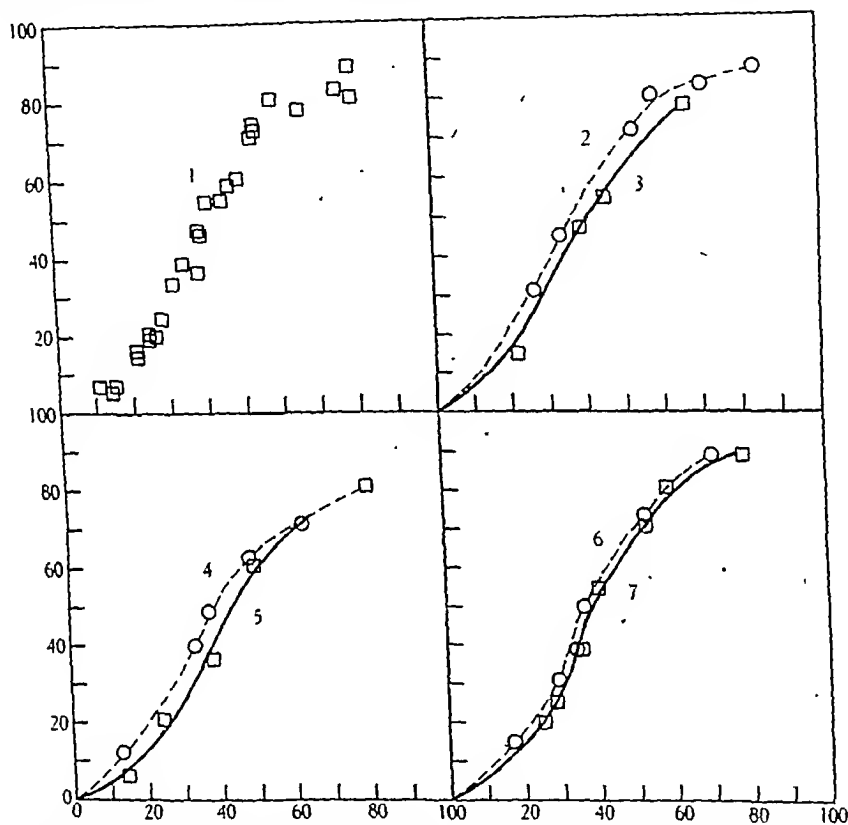
Fig. 2. Oxygen dissociation curves of suspensions of corpuscles: circles, foetal; squares, maternal. Ordinates: percentage oxygen saturation. Abscissae: oxygen pressure in mm. Hg. 1, composite curve of maternal corpuscles. Curves 2 and 3, Exp. 15. Curves 4 and 5, Exp. 16. Curves 6 and 7, Exp. 17. Oxygen capacities are recorded in Table 1.

obtained from haemoglobin preparations dialysed against isotonic buffer. The results of Exp. 5, in which $M/15$ buffer was used, are shown in the curves numbered 6 and 7. The lower electrolyte content alters the position of the curves, the 50% saturation point being moved, on an average, 8 mm. Hg to the left.

Oxygen dissociation curves on foetal and maternal corpuscles suspended in isotonic buffer solution are shown in Fig. 2. The curve numbered 1 is a composite curve of all the maternal corpuscles. The range of variation here is also

small. The curves numbered 2 and 3 and the pair 4 and 5 show the results of Exps. 15 and 16 respectively. The data of Exp. 17 are represented by curves 6 and 7. The position of the curves is now seen to be reversed and the foetal curve lies to the left of the maternal in accordance with the findings for whole blood of Leibson, et al. [1936]. This difference may be very slight and in one experiment (18) both curves appear to be identical. There is a close resemblance also between the wide range of variation in the shape of the foetal curves reported by these authors and that observed in this work. Leibson et al. describe two extreme types of dissociation curve for foetal blood with numerous intermediate forms. One type resembles the adult in shape differing only in position; while the other has a greater degree of inflexion, rising more sharply than the maternal curve which it crosses in the region of 60–70 mm. pressure. If due allowance is made for the reversal in position of the haemoglobin curves it is evident that the pair of curves numbered 2 and 3 in Fig. 1 and curves 6 and 7 in Fig. 2 conform more to the first type, while the remaining pairs of curves in Figs. 1 and 2 resemble the second type. Similar variations have been described by Barcroft et al. [1934] in foetal goat blood and by McCarthy [1933] in foetal goat haemoglobin. The difference in shape of the curves may be expressed by the calculation of n in Hill's equation, $y/100 = Kx^n/(1 + Kx^n)$, in which y represents percentage saturation, x oxygen tension and K is a constant. The value of n was determined by formula from two points covering a wide range of saturation [McCarthy, 1936] or from logarithmic curves. The average value of n for foetal haemoglobin is 2·1 with a probable error of $\pm 0·2$, and for maternal haemoglobin 2·3, with an error of $\pm 0·2$.

It remains to consider the significance of the reversal of the positions of the foetal and maternal dissociation curves determined on corpuscles and on haemoglobin solutions. The data obtained in four pairs of corpuscle suspensions and a similar number of haemoglobin solutions indicate that foetal haemoglobin undergoes little alteration in oxygen affinity on separation from the corpuscle, the average pressure at 50% saturation being reduced by less than 2 mm. Hg. The maternal corpuscle affects the behaviour of haemoglobin to a much higher degree, the average oxygen pressure at 50% saturation for maternal haemoglobin being 9 mm. Hg less than that obtained for maternal corpuscle suspensions. Hill & Wolvekamp [1936], using dilute solutions and suspensions of blood, found a similar change but stated that the apparent low oxygen affinity of human foetal haemoglobin was due to a great increase in the oxygen affinity of the corresponding adult haemoglobin on dilution. The findings in the present work, in which stronger maternal haemoglobin solutions were used, indicate that human foetal haemoglobin, unlike that of other species investigated, has a lower oxygen affinity than maternal and that this relationship is reversed in whole blood by some unknown properties of the maternal corpuscle. The data recorded in Exp. 20 indicate moreover that the

corpuscle of the non-pregnant woman resembles the maternal corpuscle in its effect on the behaviour of haemoglobin. Further discussion on this subject must be postponed until other properties of the foetal and maternal corpuscles have been investigated.

SUMMARY

1. Oxygen dissociation curves have been measured gasometrically on concentrated solutions of haemoglobin and suspensions of corpuscles obtained from human maternal and foetal blood.

2. Human foetal haemoglobin has a lower affinity for oxygen than maternal haemoglobin. This relationship is reversed in suspensions of corpuscles since the maternal and foetal corpuscles influence the behaviour of haemoglobin to different degrees.

3. The maternal corpuscle causes a marked decrease in the oxygen affinity of haemoglobin. The foetal corpuscle does not affect haemoglobin significantly in this respect.

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and a similar number of haemoglobin solutions indicate that foetal haemo-
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a much higher degree, the average oxygen pressure at 50 % saturation for
maternal haemoglobin being 9 mm. Hg less than that obtained for maternal
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suspensions of blood, found a similar change but stated that the apparent low
oxygen affinity of human foetal haemoglobin was due to a great increase in
the oxygen affinity of the corresponding adult haemoglobin on dilution. The
findings in the present work, in which stronger maternal haemoglobin solutions
were used, indicate that human foetal haemoglobin, unlike that of other
species investigated, has a lower oxygen affinity than maternal and that this
relationship is reversed in whole blood by some unknown properties of the
maternal corpuscle. The data recorded in Exp. 20 indicate moreover that the

orpuscle of the non-pregnant woman resembles the maternal corpuscle in its ffect on the behaviour of haemoglobin. Further discussion on this subject nust be postponed until other properties of the foetal and maternal corpuscles lave been investigated.

SUMMARY

1. Oxygen dissociation curves have been measured gasometrically on concentrated solutions of haemoglobin and suspensions of corpuscles obtained. from human maternal and foetal blood.

2. Human foetal haemoglobin has a lower affinity for oxygen than maternal haemoglobin. This relationship is reversed in suspensions of corpuscles since the maternal and foetal corpuscles influence·the behaviour of haemoglobin to different degrees.

3. The maternal corpuscle causes a marked decrease in the oxygen affinity of haemoglobin. The foetal corpuscle does not affect haemoglobin significantly in this respect.

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EXPERIMENTS ON THE BLOOD SUPPLY OF NERVES

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In a recent review Adams [1942] has drawn attention to the fact that there is but little experimental evidence regarding the part played by the circulation in maintaining the functions of nerves. Much work has, however, been done on the reaction of isolated nerve to asphyxia, and reference need only be made to the important paper by Gerard [1930]. It seems reasonable to assume that the facts elicited by such experiments apply also to nerve *in situ*, and that its behaviour will consequently be modified by the presence and extent of the circulation.

Almost the only direct experimental approach along these lines is that of Koch [1926] who investigated the effect of ischaemia on the current of injury, though Fröhlich & Tait [1904] had demonstrated the dependence of the excitability of nerves upon blood supply. Reference should also be made to the work of Forbes & Ray [1923] on the conditions of survival of mammalian nerve trunks. No less important, if less direct, is the evidence of Lewis, Pickering & Rothschild [1931], whose experiments, carried out with pneumatic cuffs applied to the arm of the human subject, demonstrated a centripetal spread of anaesthesia.

It is sufficiently obvious that the blood supply of nerves has a considerable functional importance. It has been our object in this work to investigate directly the effects of ischaemia upon conduction in nerve and to assess as far as possible the significance of a blood supply.

METHODS

The experiments were done on cats anaesthetized by the intraperitoneal injection of 0·7 c.c./kg. body weight of 'Dial Liquid Compound'. Experiments were carried out in a chamber at 34–36° C., and the atmosphere was almost saturated with water vapour to prevent cooling and drying of the exposed parts of the nerve. The animal was allowed to breathe air cooled by passing through a copper spiral immersed in iced water; respiratory valves ensured the correct direction of the air flow. By this means the rectal temperature was kept constant at 37° C. The nerve employed was the external popliteal division

)f the sciatic, and it was stimulated supra-maximally through silver electrodes lelivering shocks induced in the secondary of an air-cored transformer by the lischarge of a condenser through the primary. Action potentials were recorded monophasically from non-polarizable Ag-AgCl-saline-wick electrodes by means of a condenser coupled valve amplifier of conventional design, and a cathode-ray oscillograph. It is concluded that these recording electrodes are not in fact polarized by the current of injury because identical results have been obtained both with and without an input condenser. The system had an over-all time constant of 100 msec. Photographic records were made of single traverses, which were suitably timed in relation to the action potentials by means of a Keith Lucas pendulum. The rate of the traverse and its exponential character were checked with a beat-frequency oscillator.

Unless there is a statement to the contrary the nerve in each experiment was divided close to the sciatic notch and its motor branches at the knee were cut. There was thus available a conduction distance of from 20 to 25 cm. If the nerve trunk had to be exposed for experimental purposes the muscles and the skin were always sutured over the nerve and the ends of the trunk only exposed at intervals for stimulation and recording, after which they were buried again in the limb.

The record of the action potential taken at the ankle after stimulation at the sciatic notch remained constant for at least 6 hr.; it showed the *A* wave of Gasser [1934] due to impulses in the larger fibres, with a conduction rate of 35–75 m./sec. The effects of ischaemia on the *A* wave only were studied. The altered state of the nerve was reflected in the changed action potential whose height is proportional to the voltage developed; this in its turn was accurately determined by the use of a suitable calibrator.

It has been thought desirable not to lengthen the description which follows by including protocols; the experiments which are described have all been repeated several times with the same results. Where there have been variations in the times recorded in repetitions of the same experiment the extreme limits found are given in the text.

RESULTS

The sciatic nerve and its external popliteal continuation have a' well-defined blood supply. One or more longitudinal vessels run the whole length of the nerve, supplied from above by a relatively large arterial branch running on to the trunk in the gluteal region. This branch will be referred to as the blood supply from above. In addition, vascular twigs run on to the nerve at different levels in the limb and, dividing into ascending and descending branches, join the main longitudinal vessel. There is one such lateral vessel at the level of the hamstring branches of the internal popliteal nerve, another in the lower third of the thigh, one at the level of the head of the fibula, and several tiny twigs in the leg and at the ankle.

This blood supply provides a wide margin of safety for the nerve, and it was found that several of the lateral vessels could be cut without interfering with conduction; but the following experiment shows that a blood supply is essential. The supply to that part of the external popliteal nerve lying in the thigh was reduced by dividing the sciatic nerve close to the sciatic notch together with the blood supply from above. The lateral vascular twigs running on to the nerve trunk in the thigh were also cut. Therefore there remained only such blood supply as reached the nerve in the thigh along the longitudinal vessel coming up from, and supplied by, the lateral twigs in the leg. The internal popliteal nerve was cut in the leg to prevent distortion of records by a motor response to stimulation. The sciatic nerve was exposed in the upper part of the thigh and the external popliteal nerve at the ankle every 30 min. for stimulation and recording respectively. The action potential at the ankle began to fall after 2 hr. until at the end of about 6 hr. the state of the nerve was as follows. While stimulation in the upper third of the thigh was without effect, an action potential appeared at the ankle and increased as the stimulating electrodes were moved peripherally until they reached the lower third of the thigh, when a normal response was obtained. Thus the blood supply ascending from the leg along the longitudinal vessel only sufficed, after the lapse of 6 hr., to maintain the nerve in a normal state in the lower third of the thigh.

The relative importance of the blood supply from above and of the lateral vascular branches was investigated as follows. All the lateral vessels running on to the nerve between the upper part of the thigh and ankle were cut, but the blood supply from above was left intact. The external popliteal nerve was stimulated in the upper part of the thigh. At the end of 3 or 4 hr. the action potential at the ankle was 80 % of the original. The blood supply from above was now cut off. In half an hour the potential had fallen almost to zero. Control experiments have shown that the action potential does not undergo any significant change during this half hour if the blood supply from above be left intact.

It is clear that the blood supply from above must have sufficed to maintain at a high level the excitability of the nerve under the stimulating electrodes. But it is by no means so clear that conduction throughout the nerve was being similarly maintained. It is possible that it depends upon diffusion from the surrounding tissues of the limb, which are of course normally vascularized.

To explore this possibility a tourniquet was applied to the limb so as to leave out the sciatic nerve together with its blood supply from above. This was done by tunnelling through the soft tissue of the upper part of the thigh and passing two lengths of rubber tubing through the hole. These were then tightly stretched and tied around all the tissues in front of and behind the sciatic nerve. When such a nerve is stimulated above the level of the double tourniquet the action potential at the ankle disappears in 45 min.; it is still normal at the

vel of the head of the fibula in 120 min., though only a very small one remains
1 the leg. It would appear therefore that conduction ceases in a partially
evascularized nerve much earlier ·when it lies in an avascular than in a
·ascular limb.

The dependence of continued nervous activity upon the environment was
urther demonstrated by experiments in which a complete tourniquet was
applied. Three turns of tightly stretched rubber tubing were applied at the
:oot of the limb and were obviously effective. The external popliteal nerve was ·
divided distal to the tourniquet and stimulated just below the level of section.
The action potential at the ankle began to diminish in 18 min. and had fallen
to zero in 30 min. It was immaterial whether the nerve was stimulated just
below the tourniquet or at knee level, and the same result was obtained when
the tourniquet was applied just above the knee. The period of 30 min. is a very
constant one under these experimental conditions and may be called the
'ischaemia time'. Though the nerve below the knee seems to be completely out
of action it is found that a considerable action potential can be recorded in the
thigh in response to stimulation. at the sciatic notch even 70 min. after the
application of the tourniquet. The nerve at this level is of course quite a large
one and therefore not directly comparable with that used at the ankle. This
objection can be overcome by stimulating the sciatic trunk just below the
tourniquet and recording from the branch of the internal popliteal nerve to the
lower part of the biceps muscle. This nerve is somewhat more slender than that
used at the ankle, but it continues to produce an action potential for an hour
and a half after that at the ankle has disappeared. The longer survival of con-
duction in the nerve in the thigh is again suggestive of an environmental effect,
and the following experiment finally establishes it.

In a limb with a rubber tube tourniquet at its root the external popliteal
nerve was cut at the ankle and freed from its bed up to the lower third of the
thigh, where it was laid alongside the main trunk. The nerve was stimulated
just below the tourniquet and records were made, at intervals. The action
potential at the transposed ankle level disappeared in from 100 to 135 min.
The time involved is much longer than the 'ischaemia time' referred to above,
but it must be borne in mind that the nerve was exposed to the air during the
dissection. Control experiments undertaken to assess the importance of this
factor showed that when the nerve was freed from its bed and replaced in its
normal position the action potential disappeared in 50 min. (Fig. 1). The nerve
similarly freed and replaced in a limb without a tourniquet continued to con-
duct normally for at least 2 hr. This is undoubtedly an environmental effect on
conduction. It might be thought that the effect is due to reduced excitability,
but it is clear that the excitability of the ankle fibres at the point of stimulation
was maintained for nearly twice as long as the nerve survives in its normal
situation.

The environmental effect upon conduction which has been demonstrated invites further analysis. The experiments now to be described are in the nature of a preliminary exploration of the phenomenon.

A possible criticism that the longer survival in the thigh might be due to the passage of small quantities of blood under the tourniquet in particular through vessels in the bone can be met by the fact that no such blood leak can be detected when the limb is amputated distal to the tourniquet. Moreover, in an
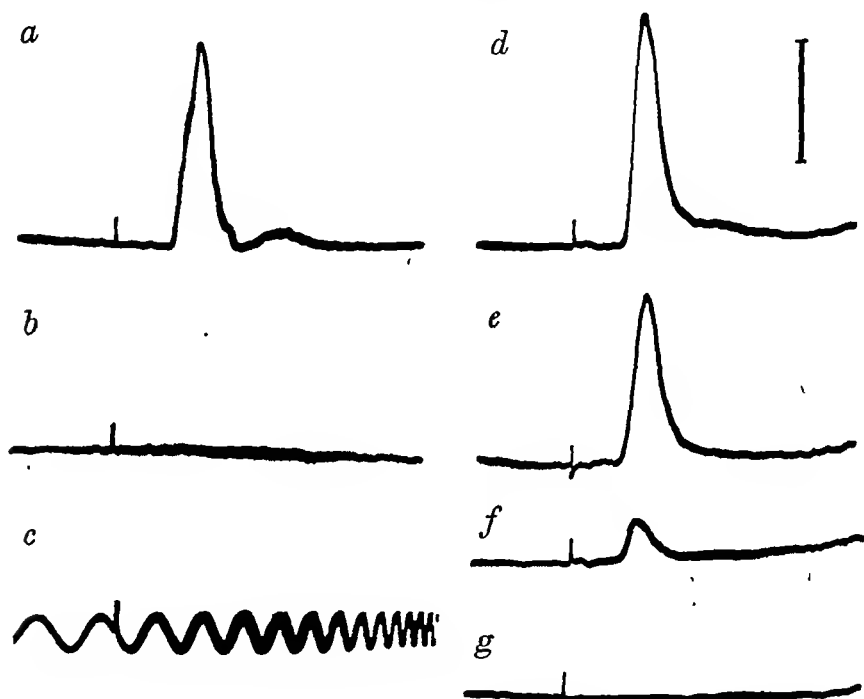
Fig. 1. (a) Left leg. Nerve freed to lower third of thigh and replaced in its bed. Record at ankle 14 min. after application of tourniquet. (b) As (a). Record at ankle 39 min. later. (c) 500 o./sec. (d) Left leg. Nerve freed to lower third of thigh and placed alongside sciatic trunk. Record at transposed ankle level 14 min. after application of tourniquet. (e) As (d). Record at transposed ankle level 36 min. later. (f) As (e). 88 min. later. (g) As (f). 106 min. later. Calibration for all action potential records in (d)=250μV.

experiment in which the limb was amputated proximal to the tourniquet the same time relations between nerve block in the leg and in the thigh were observed.

Again, it might be thought that the survival of the nerve in the thigh should be attributed to a temperature difference. Temperatures taken with a thermometer embedded in the limb under the conditions described above are higher by 1° C. in the thigh than at the ankle. Such a difference is unlikely to be significant, and in any event if it were significant the nerve at the ankle should

survive the longer. Experiments with tourniquets carried out at room temperature, but otherwise under identical conditions, show that nerve survives longer in the cold, and this confirms an observation which has already been made by Allen [1938]. The longer survival in the thigh cannot be explained by a temperature difference; it remains to be determined whether the factor concerned is exercising a beneficial influence in the thigh or an adverse one in the leg.

A rubber tube tourniquet was applied to a limb, the sciatic trunk divided immediately distal to the tourniquet and freed to knee level, along with the branch to biceps which was used for recording. The nerve was stimulated at its cut end. When the initial record had been made the nerve and its branch were wrapped in a sheet of thin rubber and laid back in the nerve bed, and the muscles and skin sutured. Thirty-five minutes later, and 1 hr. after the application of the tourniquet, the action potential had disappeared. This is a considerably shorter period of survival than is found when the nerve remains in direct contact with the tissues of the thigh. In a control experiment in which no tourniquet was applied, the action potential was little changed in 90 min.

On the other hand, the period of survival of the nerve in the leg is not altered by enclosing it in rubber tubing. For this experiment tourniquets were applied on both limbs and the nerves were freed from the ankle level to the knee. On one side the nerve was threaded through a rubber tube. Muscles and skin were then sutured over as usual. The action potential at the ankle disappeared simultaneously on both sides in about 50 min.

These experiments, in which the nerve was isolated from its environment by a rubber membrane, show that while the procedure is without effect in the leg, the survival time of the nerve in the thigh is considerably reduced. This again is probably best explained in terms of anoxia, and it is suggested that the fleshy tissues of the thigh supply oxygen to the nerve by diffusion, while in the leg the character of the tissues—predominatingly tendinous, bony, and of less bulk—does not provide such a large reservoir.

It is obvious from the experiments so far described that if nerves of the cat are to function at all there must be a supply of blood either directly or to the surrounding tissues. The impression gained is that the blood supply need not be a large one, and this is confirmed by experiments making use of a pneumatic cuff.

Such a cuff, 4 cm. wide, was applied in the middle of the thigh and surrounded with a plaster of Paris bandage to prevent slipping and stretching; it was distended with air at a pressure of 150 mm. Hg, some 30–40 mm. higher than the carotid blood pressure previously determined with a mercury manometer. The nerve was stimulated above the cuff at the sciatic notch and records were made at the ankle. Within 15 min. the action potential began to fall and

it disappeared very constantly in 25–30 min.—approximately the 'ischaemia time' referred to above. But if at this time the nerve was stimulated distal to the cuff a normal response was obtained at the ankle for at least 1 hr., after which time the pressure in the cuff was released so that recovery might be studied.

It is clear that during the application of pressure with the pneumatic cuff conduction in the nerve under it was blocked, for the distal part was shown to be functioning and the central part had not been interfered with. It seemed likely that some blood was passing into the limb in spite of the distension of the cuff. The truth of this explanation was confirmed by the oozing of blood when the tibial artery was opened. This is not surprising when it is borne in mind that fallacious results in human blood-pressure measurements may easily be obtained by the use of too narrow a sphygmomanometer cuff. In the present experimental conditions it was necessary to have the pressure in the cuff at least 50 mm. Hg in excess of the carotid blood pressure before all oozing from a severed tibial artery ceased. When the cuff was distended with a pressure of 240–280 mm. Hg, it could be shown that the action potential at the thigh disappeared in 30 min. whether stimulation was above or below the cuff. The explanation of the results obtained with the lower pressure lies in the fact that the large arteries of the limb allow some blood to pass to the periphery though the pressure under the cuff is sufficient to stop the circulation in the nerve itself and in the surrounding tissue.

Finally, some reference must be made to the recovery which ensues when the tourniquet is removed from the limb. If the circulation has been stopped for no more than 1–$1\frac{1}{2}$ hr., a small action potential can be detected at the ankle in response to stimulation in the thigh within 30 sec. of removal of the tourniquet; and this holds also in the case of the pneumatic cuff for both the lower and the higher pressures. Recovery becomes complete in 5 or 6 min., by which time it is obvious from the appearance of the limb that the circulation has been restored. When, however, the tourniquet is applied for longer periods, the recovery becomes slower and of less degree. At the same time it is clear from the appearance of the limb that the circulation is being restored more slowly and less completely. Indeed, so far as can be judged from inspection recovery of the nerve occurs *pari passu* with recovery of the circulation in the limb. From the fact that nerves of limbs to which a pneumatic tourniquet has been applied for 3 or 4 hr. have recovered completely in from 1 to 2 hr. it can be concluded that the former period in ischaemic conditions does not damage the nerve permanently.

DISCUSSION

It is well known that in experiments with isolated nerves conduction is maintained in an atmosphere of pure oxygen. Our experiments demonstrate that in the animal body a nerve requires an active circulation to maintain normal

conduction. The anatomical arrangement of the blood supply provides a wide margin of safety; but in conditions of complete ischaemia a nerve ceases to conduct in about 30 min. at 25° C. This time is in accordance with the results of other workers who have subjected nerves to anoxia or to ischaemia.

TABLE 1

Author	Nerve used	Temp. °C.	Result
Gerard [1930]	Dog peroneal *in vitro*	25	Blocked after 60 min in nitrogen
Gerard [1930]	Dog peroneal *in vitro*	28	Blocked after 24 min. in nitrogen
Lehmann [1937]	Cat peroneal *in vitro*	37	Blocked in 35 min. in hydrogen
Clark, Hughes & Gasser [1935]	Cat saphenous compressed by pneumatic cuff *in vivo*	Not stated	A and B waves blocked in 45 min.
Lewis, Pickering & Rothschild [1931]	Human arm nerves compressed by pneumatic cuff *in vivo*	35–36	Blocked after 16 to 35 min.

The time taken for cat peroneal nerve to be blocked by anoxia at 37° C. (Lehmann) corresponds closely with that taken for ischaemia to produce a block in the nerve at ankle level in our experiments, and also with the times for ischaemia block reported by the last two authors listed in the table. It does not appear likely that tourniquet ischaemia introduces any factor other than anoxaemia, for anoxia *in vitro* and tourniquet ischaemia *in vivo* produce their effects in the same time.

The small oxygen requirements of isolated nerve have led to the view that the circulatory requirements must also be small, and this view is supported by our experiments. It has been questioned of late by Bülbring & Burn [1939]. It should be noted that the experiments they describe were carried out on limbs perfused with defibrinated blood, and it would be well to exercise care in applying results obtained in this way to the problem of the normal circulatory requirement. Indeed, until there is more conclusive evidence to the contrary it must continue to be assumed that the circulatory requirements of nerve though definite are small.

It has been shown that if the hind limb of a cat be rendered ischaemic by a tourniquet applied at the root, conduction fails first in the periphery. A rather similar phenomenon was described by Lewis *et al* [1931], who showed that in the human subject the application of a pneumatic cuff or of a special clamp (designed to press on the nerve without interfering with the circulation to the limb) caused an anaesthesia which spread centrally from the finger tips. In our experiments the degree of block produced appears to be dependent upon the degree of anoxia which develops. It certainly does not depend on the length of the nerve fibre rendered ischaemic, for the 'ischaemia time' of 30 min. is unaltered when the tourniquet is applied at the level of the knee though the length of the ischaemic part of the nerve has thereby been halved. Our experiments with the pneumatic cuff show further that when the circulation to the nerve is cut off locally the peripheral part continues to conduct normally. We

are satisfied that this nerve block is not due to direct pressure. It will be recalled that Gasser [1934] has taken the same view. He showed that the block produced by direct pressure developed more slowly and that recovery is also slow, an observation which we have been able to confirm in experiments to be described elsewhere.

Our results, so far as they go, do not support the view of Lewis et al. that the nerve in the proximal part of the limb is more susceptible to ischaemia than in the distal, or that a sensory nerve is more rapidly blocked by ischaemia the farther away it is from its sensory endings. We conclude that given equal anoxaemia the nerves in the leg and in the thigh are blocked in much the same time. It is necessary, however, to bear in mind a point they have emphasized, for their 'observations were carried out upon very long nerves lying completely undisturbed by dissection in their natural surroundings and tested by natural stimuli'. The length of nerve rendered ischaemic did not exceed 4 cm. in our cuff experiments and 25 cm. in our tourniquet experiments. It may be that the explanation of the discrepancy is to be found in this fact. Indeed, our observations are much more in accord with their 'uniformly developing anaesthesia resulting from pressure rendering short stretches of nerve trunks ischaemic...'.

SUMMARY

1. The blood supply of the external popliteal nerve of the cat consists of a branch running down the nerve from above and of lateral branches running on to it at approximately constant levels.

2. A small though definite blood supply is essential for the maintenance of conduction in the nerve. The anatomical arrangement of the blood supply provides a wide margin of safety. Even if all the lateral branches are divided conduction in the nerve is unaffected in a normally vascularized limb. In a limb rendered avascular by a tourniquet from which the arterial branch to the nerve from above is excluded, conduction is maintained in the nerve to the upper part of the leg. Below this level the nerve is inactive.

3. In a limb rendered ischaemic by a complete tourniquet at its root the nerve in the leg and foot becomes inactive in about 30 min., though the nerve in the thigh survives for at least two hours.

4. If in such a limb the nerve in the foot and leg be freed from its bed and laid alongside the main trunk in the thigh, it survives much longer in the transposed situation than in the normal one. The effect cannot be attributed to a difference of temperature, or to leakage of blood under the tourniquet.

5. The survival time of the nerve in the thigh may be curtailed by enclosing it in rubber, a procedure which has no effect on the survival time in the leg. It is suggested that a greater supply of oxygen is available in the thigh to supply the nerve by diffusion.

6. A pneumatic cuff, 4 cm. wide, applied to the limb and distended to a pressure equal to that in the carotid artery interrupts conduction in the nerve immediately under it in 30 min. Conduction in the nerve distal to the cuff is unaffected. This state of affairs must be due to local ischaemia. The pneumatic cuff does not become a completely effective tourniquet till it is distended to at least 50 mm. Hg in excess of the carotid pressure. Under such conditions conduction fails also in the nerve distal to the cuff.

7. On the release of a tourniquet recovery begins in 30 sec. and is complete in 5 or 6 min. if the circulation has been interrupted for no more than $1\frac{1}{2}$ hr. Recovery is slower after longer applications but the nerve is able to recover from ischaemia of 4 hr.'s duration produced by a pneumatic cuff. It is suggested that the factor governing recovery is the return of the circulation.

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THE EFFECTS OF PRESSURE ON CONDUCTION IN PERIPHERAL NERVE

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Though relatively slight pressure on a nerve can stop conduction, it only does so when applied locally. Normal nervous activity is possible under conditions of generally increased pressure such as exist on the sea bed, and it has been shown experimentally [Ebbecke & Schaefer, 1935; Grundfest & Cattell, 1935] that if a nerve be totally enclosed in a pressure chamber conduction only begins to fall at pressures between 6000 and 8000 lb./sq. in.

A review of the literature reveals numerous reports of experiments designed to demonstrate the effects of pressure applied locally. Many investigators have, however, confined their attention to isolated nerves, and their methods have been so various that results are not strictly comparable. The beautifully simple experiments of Weir Mitchell [1872] showed that a pressure of a column of mercury 18–20 in. high interrupted conduction in the sciatic nerve of the rabbit in 20–30 sec. The principle of applying fluid pressure was further developed by Meek & Leaper [1911] and later by Gasser & Erlanger [1929], who used electrical methods for recording nervous activity and were able to show that a pressure of 25 lb./sq. in. blocked conduction in frog's sciatic nerve in a few minutes and that the larger fibres were affected first.

It is clear that in nerves with intact circulation the effects of pressure might be due to deformation or to ischaemia or to both. The experiments of Gasser & Erlanger [1929] suggest the former explanation; and there is direct evidence that the deformation caused by local pressure is most severe at the boundaries of the compressed part [Edwards & Cattell, 1928]. On the other hand, the experiments of Lewis, Pickering & Rothschild [1931] on man and those of Clark, Hughes & Gasser [1935] on animals showed that with quite low pressures a nerve in a limb may be blocked in from 16 to 40 min., and that ischaemia was the mechanism involved. These experiments have been confirmed by us [Bentley & Schlapp, 1943].

In man it is not uncommon to find that localized pressure of some hours' duration over a nerve trunk results in a temporary paralysis. Whether such a

block is due to ischaemia or to the direct effects of pressure is not clear, and it is the purpose of the experiments described in this paper to inquire further into the relation between pressure and ischaemia in producing a block to conduction.

METHODS

The experimental procedures as regards the preparation, stimulation and recording of nervous activity have already been described [Bentley & Schlapp, 1943]; there remains the method used to apply pressure directly to nerves.
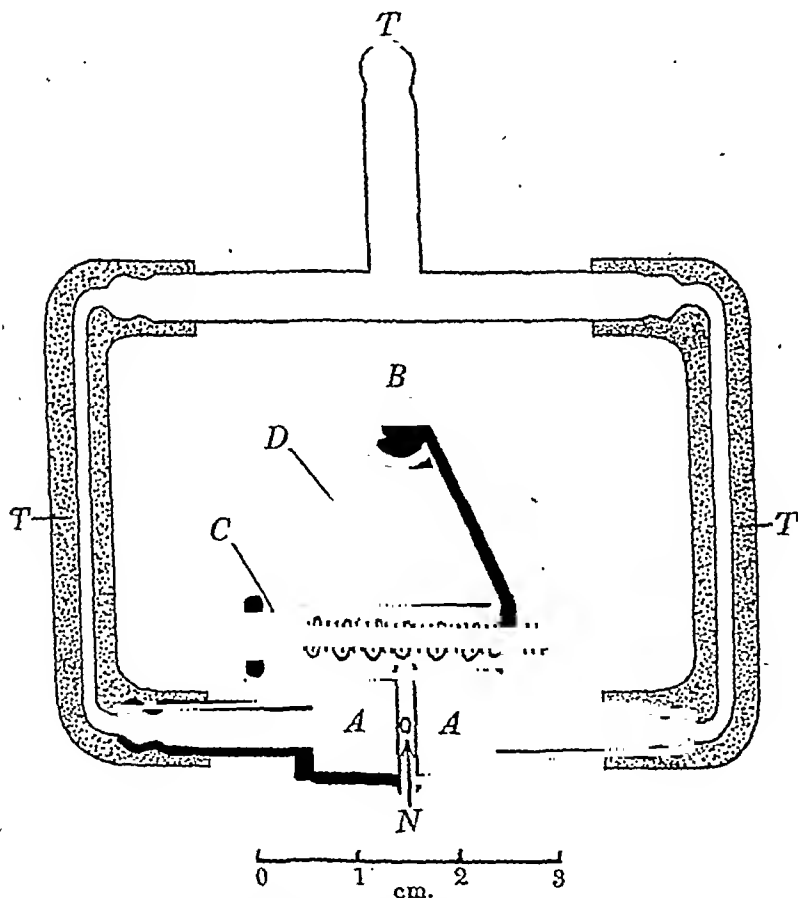
Fig. 1. Sectional view of apparatus for compressing nerves. *AA*, brass chambers hinged at *B* and covered with rubber membranes between which the nerve *N* is placed. *C* is an adjusting screw and *D* a stop to prevent *too close approximation*. The chambers are supplied with air at the required pressure through the tubes *T*.

In principle it consists in placing the nerve trunk between pneumatic cushions at a fixed distance apart. These are inflated to the required pressure by means of an air pump. The constructional details of the apparatus are shown in Fig. 1.

A calibration was obviously desirable in order that the actual pressure applied to the nerve might be known. The apparatus (*A*, Fig. 2) was therefore applied to an excised piece of the external jugular vein of a cat. The ends of the vein were tied to small glass tubes leading to a manometer (*B*) on the one hand and a tap (*C*) on the other. The vein and the tubes were filled with Ringer's solution and the pneumatic cushions (*A*) distended to a known pressure of up to 150 mm. Hg. The manometer (*B*) was filled with mercury so that a rather lower pressure was developed in that part of the vein connected to it. With the tap (*C*) open the pressure in the pneumatic cushions was now slowly released and the mercury meniscus (*D*) observed for movement, which would show
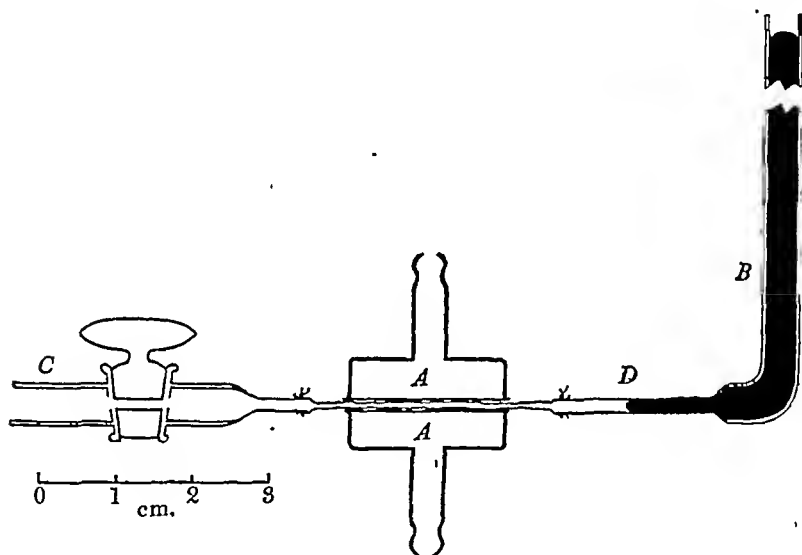
Fig. 2.

that fluid was passing through the vein. This invariably happened when the cushion and manometer pressures were within 5 mm. of each other. It may therefore be assumed for practical purposes that the pressure in the cushions is transmitted directly to the nerve.

The apparatus was used in two forms, 2 and 4 cm. long respectively, but they gave identical results. In applying the apparatus the necessary length of nerve was freed in the lower third of the thigh, slightly raised on glass hooks, the cushions adjusted on either side and distended so that the nerve was held between them. The glass hooks were removed, the skin sutured above and below the apparatus, and a gauze swab soaked in saline placed over it. The external popliteal nerve was stimulated in the upper part of the thigh and records were made from the nerve at the ankle. The effects of pressure on conduction were studied in the '*A*' fibres only [Gasser, 1937].

RESULTS

Preliminary experiments showed that with applied pressures of 10–15 mm. Hg the size of the action potential remained constant for at least 6 hr. With pressures between 130 and 200 mm. Hg however, it became reduced in 40 min., indicating that conduction had ceased in a number of fibres. The block continued to develop and was complete or almost so in 2–3 hr. As the action potential became reduced the first part of the 'A' wave declined more rapidly than the last, showing that conduction failed first in the fibres with the fastest conduction rate, or in other words, those of larger diameter [Gasser, 1937], though a complete separation of fast and slow fibres was not seen. The part of the nerve below the pneumatic cushions was unaffected, as a normal response was obtained throughout the experiment when stimulation was below the block.

In these experiments the pressure applied was made approximately equal to the carotid blood pressure, as the block was at first interpreted in terms of anoxia consequent on ischaemia. Nevertheless, an attempt to restore conduction during the period of decline by the intravenous injection of 1 mg. of adrenaline hydrochloride or of 10 units of posterior pituitary extract failed, though the procedures markedly increased the blood pressure. Conversely, attempts to hasten the onset of block by repetitive maximal stimulation for periods of 6 min. at 65 per sec. and 33 min. at 170 per sec. were unsuccessful. The action potential was indeed somewhat reduced during stimulation, but recovered fully within half a minute of its cessation.

The results of twenty-seven experiments with various pressures are reproduced diagrammatically in Fig. 3; they have been arranged so that the applied pressures—represented for each experiment by a black column—form an ascending series from 10 to 200 mm. Hg. The associated white column gives the magnitude of the carotid blood pressure in each case. The results fall into three groups. In the first (A) the action potential was affected only very slightly or not at all after 2½–3 hr. The pressure applied was up to 100 mm. Hg, a pressure less than that of the blood in the carotid artery. In the second group (B) there was partial disappearance of the action potential in 3 hr., indicating partial block. The applied pressure in this group was 120 mm. Hg, somewhat greater than that in the carotid. In the experiments of the third group (C) the nerve was found to be completely blocked or almost so in 2½–3 hr., and the applied pressure was between 130 and 200 mm. Hg. In all but two of the experiments of this group the blood pressure was less than the applied, and if these results were for the moment neglected it could be said that nerve block occurs only when the applied pressure exceeds that in the carotid artery. These two experiments, however, show that a nerve may be blocked by the application of a pressure less than that prevailing in the carotid artery. The results

taken as a whole suggest that there is a critical value of about 130 mm. Hg for the production of nerve block by pressure within the time limits used.

When the pressure is released the subsequent behaviour of the compressed part differs in the three groups. In the first (A), in which conduction had been affected only slightly or not at all, no further change occurred in the next 2 hr. In the second group (B), where there had been partial block, one nerve recovered and the other progressed to complete block in 2 hr. In the third group (C), in which block was complete or almost so, there was no recovery after 2 or
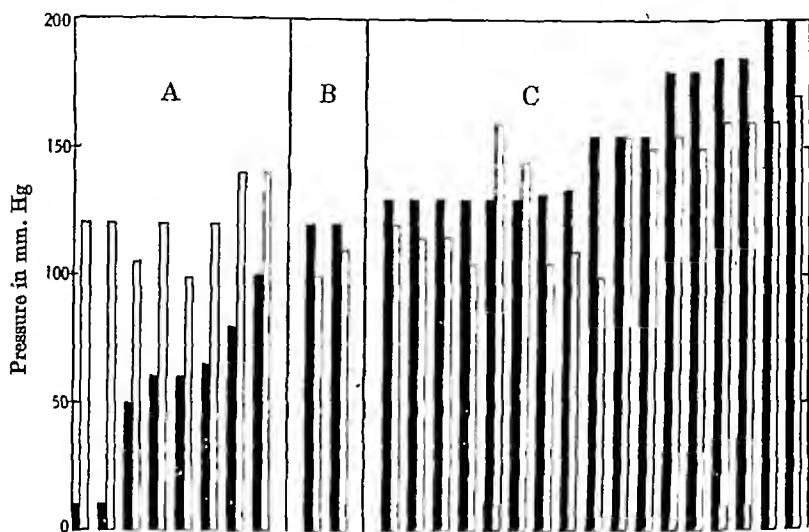
Fig. 3.

3 hr. Indeed, the reverse of recovery was sometimes observed. In five experiments the small potential remaining when the pressure was released disappeared in the course of half an hour just as though the pressure had been continued. In one case where the observation was continued for 13¾ hr. after the establishment of complete block the action potential recovered to about 30 % of its original height.

In eight experiments in which block was complete or almost so, the nerve was explored after removal of the pneumatic cushions by moving the recording electrodes centrally while stimulating at the top of the thigh as usual. The following results were obtained:

Situation	Action potential
In mid-leg	Absent or minute
One cm. below lower edge of compressed part	Small—200 μV. (normally 2 mV.)
A few mm. above lower edge of compressed part	Slightly larger—300 μV.
In middle of compressed part	Considerable increase—1·5 mV.
A few mm. below upper edge of compressed part	Larger—2 mV.
One cm. above compressed part	Still larger—3 mV.

These results have been expressed diagrammatically in Fig. 4.

There was thus a sudden increase in the action potential in the lower part of the compressed length, a further increase as it was traversed, and another considerable one just above it. As it was thought that this result might be dependent on some local anatomical feature, an experiment was performed in which pressure was applied to the nerve in the middle of the leg. An identical result was obtained.

The block is thus mainly situated at the extremities of the compressed part, and it is here that Calugareanu [1901] and Edwards & Cattell [1928] showed that deformation and displacement of nerve substance were most severe.

Various changes were visible to the naked eye in the nerve when the pressure was released. The surface of the compressed part was moist, and if there was a film of blood over it this was bright red in colour. The nerve substance
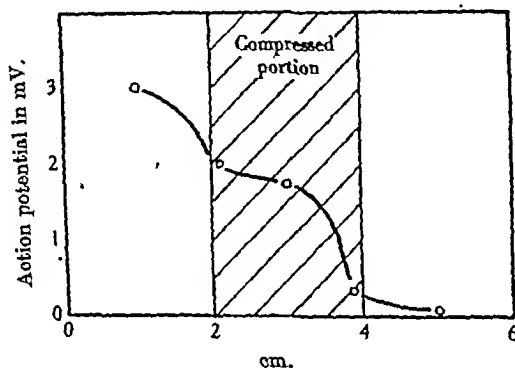
Fig. 4.

of the compressed length was rather pale and somewhat flattened; and the blood vessels on its surface were sometimes not obvious at all, sometimes pale red, and sometimes of a brownish hue. The vessels on the remainder of the nerve, though occasionally congested, showed no other abnormality. It was not possible to relate the appearance of the superficial blood vessels to the state of conduction in the nerve.

Immediately after removal of the pneumatic cushions in experiments in which conduction had been abolished, the superficial blood vessels of the compressed part were seen to fill slowly from above and below. This reflux did not give the impression of an active circulation.

In order to inquire further into the circulatory state a solution of Evans blue was injected intravenously. This dye remains in the plasma and does not normally leave the circulation for many hours. In the concentration used (20 mg./kg.) its presence was obvious from the deep blue colour which appeared in the superficial vessels of the external popliteal nerve within 10 sec. of the injection. Obviously the blue colour can only develop in vessels in which blood is circulating.

In experiments in which, following the release of pressure, conduction was maintained completely (group A) or in part (one experiment of group B), injection of the dye showed that there was an active circulation in the previously compressed part, as the epineural vessels became deep blue in colour within a few seconds. There was often some blue staining of the nerve substance itself, and it is thought that this appearance of the dye outside the blood vessels indicates that they have been damaged in the regions where staining occurred.

In experiments in which the nerve was still blocked 2 or 3 hr. after the pressure had been released (one experiment group B, and all experiments group C) the injected dye showed in the longitudinal vessels of the nerve up to both edges of the compressed part, but the vessels of the latter remained red. The difference was a very striking one, and the sharp line of demarcation corresponded accurately to the limits where pressure had been applied. It was concluded that the early filling of the vessels after the release of the pressure was in fact a passive reflux and that there had been no active circulatory return.

In view of what has been said regarding the situation of·the block, it was interesting to observe that there was considerable blue staining of the nerve substance above and, to a greater extent still, below the compressed part. The staining extended for about 1 cm. on either side of it and also for a few mm. into it.

It might be assumed from these experiments that the failure of conduction in the compressed part is due to local ischaemia. Yet an applied pressure of 60–100 mm. Hg should have been sufficient to stop the circulation in the small vessels of the nerve trunk where the blood pressure might well be of the order of one-half of that in the carotid. Indeed, it was possible to show experimentally that local ischaemia was not responsible for the development of nerve block.

In two experiments pressures of 60 and of 65 mm. Hg respectively were applied to the nerve for 2¾ hr. No failure of conduction was observed. Fifteen minutes before the pressure was released Evans blue was injected intravenously. Within a few seconds the dye was visible in the superficial blood vessels of the nerve up to the edge of the pneumatic cushions. Just before the pressure was released ligatures were tightly tied round the nerve trunk above and below in order to stop the circulation, and the pneumatic cushions removed. When the compressed part was examined immediately afterwards there were sharp lines of demarcation between the red vessels of the compressed part and the blue ones above and below it. Thus in spite of the stoppage of the circulation, conduction through the compressed part had persisted.

It has been shown elsewhere [Bentley & Schlapp, 1943] that conduction ceases in about 30 min. in the nerve of a limb rendered anoxic by the application of a tourniquet. It was shown above that a pressure of 60 mm. Hg applied to a nerve trunk may produce ischaemia in portions 2 or 4 cm. long and yet con-

duction is uninterrupted in $2\frac{3}{4}$ hr. The compressed part could not therefore have been anoxic. There seem to be only two ways in which it could have effected gaseous exchange—through the rubber membranes of the pneumatic cushions or by diffusion from the normally vascularized nerve above and below it.

The first explanation is to a high degree unlikely, and it was excluded by distending the pneumatic cushions to a pressure of 185 mm. Hg with nitrogen containing 5% of carbon dioxide. The nerve was not blocked until 2 hr. 40 min. had elapsed. This time is the same as that found when the apparatus was
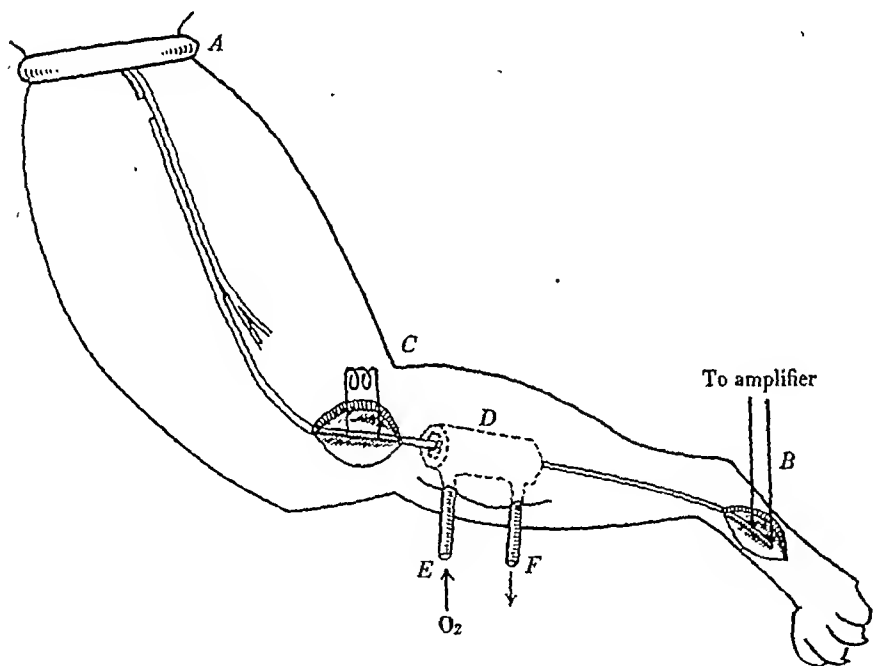
Fig. 5.

distended with air, and does not indicate that diffusion of oxygen or of carbon dioxide through the rubber membranes of the pneumatic cushions plays any part in maintaining activity in the compressed nerve.

The other explanation was much more acceptable, especially when the well-known experiments of Kato [1924] regarding the diffusion of anaesthetic vapours along isolated nerves are borne in mind.

The diffusion of oxygen along anoxic nerves was investigated as follows (Fig. 5). A tourniquet (A) was applied at the root of the limb, the external popliteal nerve cut at the ankle (B), freed up to the level of the knee (C) and threaded through a rubber tube 4·5 cm. long (D) and which was provided with side tubes (E, F). The ends of the tube D were plugged with vaseline and the

skin sutured over the nerve and tube. The side tubes, which were allowed to protrude from the limb, were used to pass a stream of oxygen along the nerve, the inflow and outflow being checked by bubbling it through water.

One hour after the application of the tourniquet and 50 min. after the oxygen supply was begun, the nerve was stimulated at the level of the knee 2 cm. above the tube (C). The action potential at the ankle 5 cm. below the tube was 80–85 % of that which had been recorded immediately after the application of the tourniquet.

It will be recalled that in a limb with a tourniquet in which the external popliteal nerve has been freed from its bed and replaced, conduction ceases in about 50 min. [Bentley & Schlapp, 1943]. The experiment described above shows that oxygen diffuses along the nerve in such a way as to maintain almost full activity over a length of 5 cm. It receives confirmation from a repetition using air instead of oxygen. As might be expected, the influence did not extend so far. The section potential was 75 % of the normal size 2 cm. below the air tube and fell to 25 % in the adjacent 2 cm.

DISCUSSION

It is clear that pressure may be applied locally to a nerve in such a way as to render it ischaemic without impairing conduction. Our experiments lead us to the conclusion that with applied pressures between 60 and 100 mm. Hg the maintenance of conduction in an ischaemic compressed portion of nerve, up to 4 cm. long, is due to the diffusion of oxygen from the neighbouring vascularized parts of the nerve. The observation that rapid stimulation of the nerve for ½ hr. did not accelerate the onset of block supports the view that the nerve had a means of replenishing its oxygen.

When pressures between 100 and 200 mm. Hg are applied, however, partial or complete failure of conduction results. It is conceivable that oxygen diffusion ceases when the applied pressure exceeds 100 mm. Hg, thus rendering the nerve truly anoxic; on the other hand, the pressure may act directly on the nerve. The first explanation seems unlikely on theoretical grounds, and there is evidence in support of the second view:

(1) The situation of the block at the extremities of the compressed part where deformation and displacement are known to be most severe is very suggestive of mechanical injury. We believe that the failure of the circulation to return when the pressure is released is due to concomitant injury of the blood vessels which is made evident by the considerable extravasations of dye which occur in these situations.

(2) The time taken for the establishment of nerve block by direct pressure is much longer than that observed in ischaemia.

(3) Recovery did not occur within 3 hr. of the release of a pressure which had produced complete block, and where the block had been incomplete it usually

became complete subsequently. This is the reverse of what happens after ischaemia, where we have shown [Bentley & Schlapp, 1943] that recovery may occur when the nerve has been in an ischaemic condition for up to 4 hr.

It is evident from these experiments and from those on ischaemia that pressure applied to a nerve can produce block to conduction in two different ways:

(1) If a pressure of 130 mm. Hg or more is applied directly to a portion of nerve 2 or 4 cm. in length, the mechanical effects—probably deformation of nerve substance at the boundaries of the compressed part—will produce a block to conduction. The block only becomes apparent in 40 min. and does not become complete for 2 or 3 hr. When the pressure is released there is no recovery during the next few hours—indeed, the block, if incomplete, may become more severe.

(2) If the pressure is applied through the full thickness of the limb by means of a pneumatic cuff, conduction ceases in about 30 min. as a result of anoxia of the nerve, and recovery after release of the pressure occurs when the circulation is restored even though a pressure of 250 mm. Hg has been maintained inside the cuff for 3 hr.

The difference between the effects of direct and indirect pressure is probably associated with differences in magnitude and distribution of pressure under a 4 cm. pneumatic cuff as compared with pressure directly applied to the nerve.

It is not known whether the block that results from the application of direct pressure would recover in a few days, i.e. a transitory block, or would proceed to Wallerian degeneration. The partial recovery which occurred in $13\frac{3}{4}$ hr. is suggestive of the former view.

SUMMARY

1. A method of applying pressure directly to nerves in the anaesthetized animal is described.

2. It is shown that pressures up to 100 mm. Hg do not produce block in $2\frac{1}{2}$–3 hr. At a pressure of 120 mm. of mercury there is some interference with conduction, while with pressures between 130 and 200 mm. Hg a block is first apparent in about 40 min. and is complete or almost so in $2\frac{1}{2}$–3 hr.

3. Repeated stimulation of the nerve does not accelerate the onset of block, and raising the blood pressure by injection of adrenaline or of posterior pituitary extract does not retard it.

4. Recovery does not occur within 3 hr. of the release of a pressure which has produced complete block, but in one experiment observed for $13\frac{3}{4}$ hr. conduction recovered in about one-third of the fibres. Where the block is incomplete recovery may occur or it may become complete subsequently.

5. There is a critical value of about 130 mm. Hg for the production of nerve block if the pressure is applied for a period of 2–3 hr. This pressure is not related to the carotid blood pressure.

6. Pressure block has different characteristics from block due to ischaemia. Thus the time taken for the establishment of nerve block by direct pressure is much longer than that observed in ischaemia; and the absence of recovery within a few hours of the release of pressure is the reverse of what happens after the relief of ischaemia.

7. A pressure of 60 mm. Hg will render a 4 cm. length of nerve ischaemic, but does not affect conduction. Although the compressed part is ischaemic it obtains oxygen by diffusion from the adjacent vascularized nerve.

8. The block produced by pressure is mainly situated at the extremities of the compressed part, where deformation is most severe.

9. The deformation is associated with injury to the nerve vessels, and absence of recovery after release of pressure is accompanied by failure of return of the circulation in the vessels of the compressed part. The circulatory failure, however, is not an important factor in the production of pressure block.

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DETERMINATION OF THE OXYGEN-COMBINING POWER OF BLOOD WITH THE BARCROFT DIFFERENTIAL MANOMETER

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The methods available for determining the oxygen-combining power of blood by gasometric measurement, using ferricyanide, fall into two groups: those in which the gas is removed from neutral solution by a partial vacuum, as in the Van Slyke [1918] apparatus, and those in which the reaction takes place at atmospheric pressure in alkaline solution, as in the various modifications of the Haldane [1898] method. The methods belonging to the second group are perhaps less used now than formerly because of the great precision of measurements with the Van Slyke apparatus, and because there are grounds for doubting the accuracy of the absolute values obtained with them. It seemed that the Barcroft manometer might be of service in cases where the highest accuracy was not required, as the technique is simple, few reagents are required, and determinations on several samples of blood may be made at one time.

A number of comparisons of the two types of apparatus have been made, several of which are discussed by Bierring, Nielsen & Nielsen [1937]. There is general agreement that the Haldane apparatus gives results 5–7% lower than the blood gas pump or the Van Slyke apparatus. Various suggestions have been put forward to account for this difference; thus Haldane [1898] considered that incomplete haemolysis and bacterial contamination were responsible, while Litarczek [1928] suggested it was due to the absorption of oxygen by reducing substances in the lipoid fraction of the plasma in the interval between the addition of the ferricyanide and the taking of the final reading. He showed that ferricyanide had a catalytic effect in bringing about this absorption of oxygen. On investigation, it was found that while blood plus ferricyanide did absorb oxygen more rapidly than blood alone, the increase in rate was not great enough to account for the differences observed.

EXPERIMENTAL

Standard Barcroft manometers were used as described by Dixon [1934], the constants being determined both by calculation from the formula of Dixon [1934], and by observation of the oxygen uptake due to the oxidation of ferrous hydroxide to ferric hydroxide by molecular oxygen [Harrison, 1933]. Good

agreement was found between the two methods. The water-bath for the mano-
meters was at room temperature, the constants of the manometers being
altered in accordance with the assumption that they varied inversely with the
absolute temperature, over the small range of temperature involved. Oxalated
ox blood was employed throughout, being used either on the day of collection
or on the next day. It was stored in a refrigerator. 0·4 % ammonia was pre-
pared by diluting 4 parts of ammonia of specific gravity 0·880 to 1000 parts
with distilled water.

The effect of 0·4 % ammonia on apparent oxygen evolution

Whole blood and thrice-washed corpuscles laked with an equal volume of water
and again centrifuged were used. 1 ml. blood or laked corpuscles and 2 ml.
distilled water or 0·4 % ammonia were placed in the working flask, with corre-
sponding amounts of water or ammonia in the control flask. CO_2 was absorbed
with filter paper moistened with $2N$ KOH in the usual way, and 0·2 ml.
saturated $K_3Fe(CN)_6$ added from a Keilin tube when required.

The apparatus was shaken at 120 oscillations per minute at room tempera-
ture for 20 min., the taps closed and an initial reading taken (zero time). The
ferricyanide was added and shaking continued for 20 min. Readings were
then taken at 10 min. intervals for 40 min. The results were plotted and the
graph obtained extrapolated to zero time. This procedure was adopted because
it corrected the results for absorption of oxygen, and because it allowed suf-
ficient time for the absorption of all CO_2. The results are shown in Table 1. It
will be observed that the results with washed cells are $2\frac{1}{2}$ % lower in the pre-
sence of ammonia than in neutral solution, while the results with whole blood
are 7 % lower.

TABLE 1. Oxygen evolved. Volumes per cent

	Neutral A	0·4 % ammonia B	$\frac{100\,B}{A}$
Whole blood	22·0	20·7	94·0
	21·1	19·3	92·5
	18·2	17·3	95·0
Washed haemolysed red cells	13·1	12·8	97·8
	21·2	20·5	96·4
	17·7	17·3	97·0

Each figure is the mean of four determinations.

To see if this difference was due to failure to set free all the combined oxygen
or to incomplete oxygenation of the haemoglobin in alkaline solution, experi-
ments were made in which the ferricyanide was dissolved in 4 % ammonia and
added to blood diluted with distilled water. The differences were found to
persist.

The results found by carrying out the reaction in neutral solution were com-
pared with those given by the Van Slyke manometric apparatus used according
to the technique of Sendroy [1931] and are given in Table 2.

Table 2. Volumes per cent

Van Slyke	Barcroft		Mean Van Slyke	Mean Barcroft
23·05	23·2	22·8	23·10	22·98
23·15	22·8	23·1		
19·13	19·5	18·8	19·02	19·17
18·90	19·1	19·3		
21·14	21·0	21·0	21·04	21·08
20·94	21·3	21·1		
18·90	18·6	19·2	18·87	18·80
18·85	18·9	18·5		
22·30	22·7	22·7	22·40	22·68
22·50	22·6	22·7		
19·15	19·4	19·0	19·12	19·18
19·08	19·4	18·9		
		Averages	20·59	20·65

The absorption of oxygen by plasma

Plasma was obtained by centrifuging oxalated ox blood, portions of 2 ml. being placed in water or ammonia as required. Water was placed in the control flasks. The final pH of the mixtures were determined with a Lovibond comparator. Wide variations were found from sample to sample, but, in twelve experiments, it was found constantly that the rate of oxygen uptake was much increased in alkaline solution. The result of one such experiment is shown in Fig. 1, which shows the apparently autocatalytic nature of the reaction and also that the amount of oxygen absorbed in the first 10 min. period is somewhat greater than that absorbed in the succeeding equal intervals of time. It is suggested that this initial rapid absorption of oxygen is at least a partial cause of the low results obtained with the Haldane method, for such an absorption occurring at the same time as the evolution of oxygen from the blood could not be observed or compensated for by backward extrapolation or any very feasible form of control. After these experiments had been completed, it was found that Pickworth & Woodhouse [1930] had observed a similar acceleration of oxygen absorption by plasma in alkaline solution.

Szgeti [1940] found that methaemoglobin appeared in whole blood diluted 1 : 500 with 0·4 % ammonia at the rate of about 3 % per hour on standing at room temperature. As it seemed possible that such methaemoglobin formation might occur under the conditions used, 50 ml. of blood was diluted with 100 ml. of ammonia and the solution allowed to stand at room temperature. The oxygen capacity remained unchanged for 6 hr.

DISCUSSION

The question of complete haemolysis was not closely investigated because the resistance of the corpuscles of various species differs. It should be noted that if saponin is to be used, it should be added before the ferricyanide, not with it, because the addition of saponin alone to blood suspended in normal saline

resulted in the evolution of about 1·2 vol. % of gas. This gas was thought to be set free as a result of the uptake of water by the haemoglobin released from the corpuscles. Much less gas was set free when undiluted blood was laked by saponin.
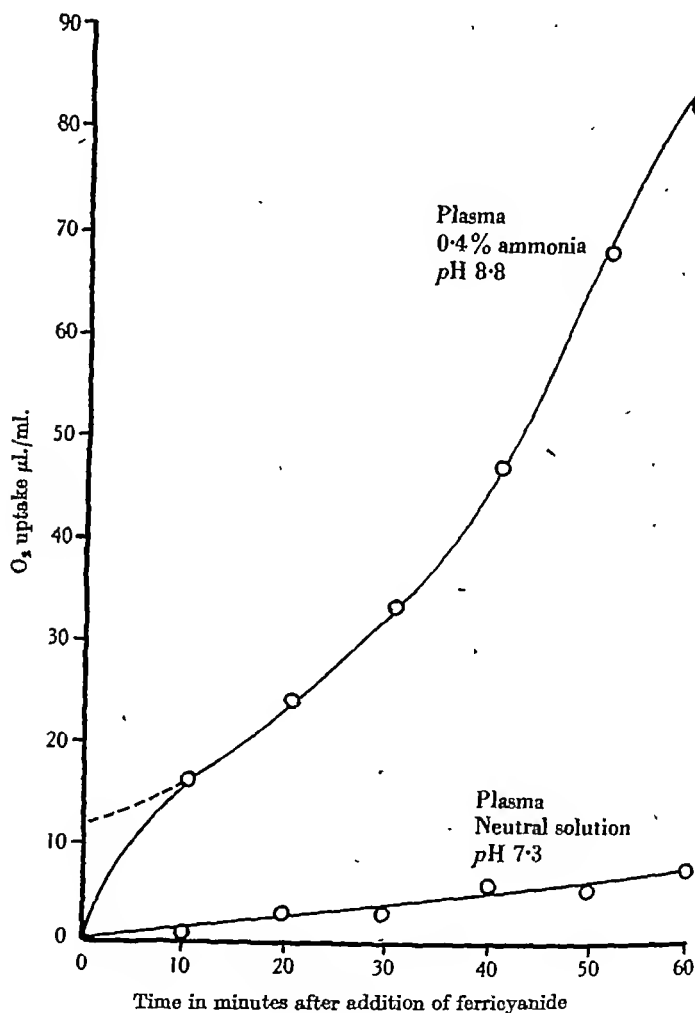
Fig. 1.

It seems probable, from the results given in Table 2, that, under the conditions specified, the same quantity is being measured with the Barcroft manometer as with the Van Slyke apparatus. With regard to the accuracy of the results, the standard deviation, calculated from thirty readings, was found to be 0·24 vol. %, so that any single determination may reasonably be expected to be within 0·5 vol. % of the true value. It is thought, therefore, that when

such a degree of accuracy would suffice, the Barcroft manometer may be of service because of the ease of its manipulation and the possibility of making multiple determinations simultaneously.

Summary

1. A method is described by which results with the Barcroft manometer may be obtained which agree, within the limits of error, with those given by the Van Slyke pump.

2. It is confirmed that less oxygen is evolved from blood by the action of ferricyanide in the presence of weak ammonia than in neutral solution, and an explanation of the difference is suggested.

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PERIODIC CHANGES IN RESPIRATORY DEPTH, PRODUCED BY CHANGES IN THE LUNG

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It is generally assumed that periodic changes in respiratory depth are produced by periodic alterations in the activity of the respiratory centre. It is the purpose of this communication to show that periodic breathing may in some cases be caused by periodic changes in the distensibility of the lungs, without any changes in the activity of the respiratory centre.

METHODS

It has been shown by Christie & McIntosh [1934] that changes in the distensibility of the lungs can be measured *in vivo* by means of simultaneous recordings of the tidal air and intrapleural pressure. The fall in pleural pressure which occurs on inspiration represents the force exerted in the lungs by the respiratory muscles, and the tidal air the expansion which results. Any increase in the activity of the respiratory centre will increase respiratory effort, and will therefore increase both the pleural pressure fluctuation and the tidal air. Changes in the distensibility of the lungs, however, will not produce any change in the pleural pressure; they will only produce a corresponding change in the tidal air. From such a record, therefore, it is possible to distinguish changes in respiratory depth due to changes in the activity of the respiratory centre from those due to changes in the distensibility of the lung. The validity of this method of measuring the distensibility of the lung *in vivo* has been established by several investigators, and is an essential part of this communication [Paine 1940; Christie, 1934; Christie & Meakins, 1934].

Observations have been made on fifteen animals—mostly cats—in which periodic breathing occurred. The technique of measuring and recording the pleural pressure fluctuation by means of a water manometer has been previously described, and the tidal air was recorded using a closed circuit chain counterbalanced recording spirometer with mercury cap valves [Christie & McIntosh, 1934]. The resistance of the circuit with the animal breathing quietly was 2–4 mm. H_2O. All animals were breathing 100 % oxygen during the whole of the experiment. Three typical experiments are described.

Results

In Fig. 1 the periodic waxing and waning of the tidal air is well shown, but the pleural pressure fluctuations remain unaltered during this period. The force exerted on the lungs thus remains constant, but the tidal air varies, and this must be interpreted as being due to a periodic change in the distensibility of the lungs and not to any change in the activity of the respiratory centre. The contrast between this type of respiratory change and that produced by alterations in the discharges from the respiratory centre is shown also in this tracing. At A, the animal takes a few irregular breaths, as shown in the tidal air tracing,
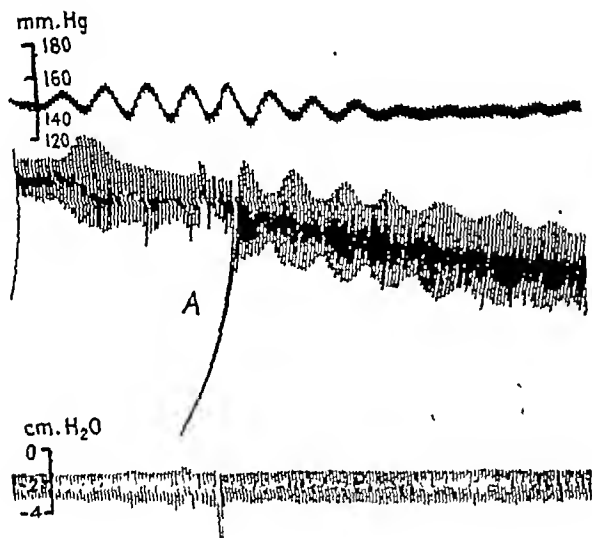
Fig. 1. Cat 52. 5·25 kg. Intraperitoneal dial 45 mg./kg. Upper tracing, blood pressure; middle tracing, tidal air; lower tracing, pleural pressure fluctuation. Time marker, 10 sec. Spontaneous periodic breathing as soon as animal was anaesthetized. Periodic variations in tidal air with no change in the pleural pressure fluctuation.

and there is a corresponding alteration in the pleural pressure fluctuation. The blood-pressure record shows Traube-Hering waves of the same periodicity as the tidal-air variations. Periodic breathing, however, persists when Traube-Hering waves are hardly visible, so that the respiratory changes do not appear to be secondary to the changes in blood pressure.

Fig. 2 also shows a marked fluctuation in the tidal air, this time of longer cycle, but again there is no change in the pleural pressure fluctuations. In this animal the blood pressure remained unaltered during the period of variation in respiratory depth.
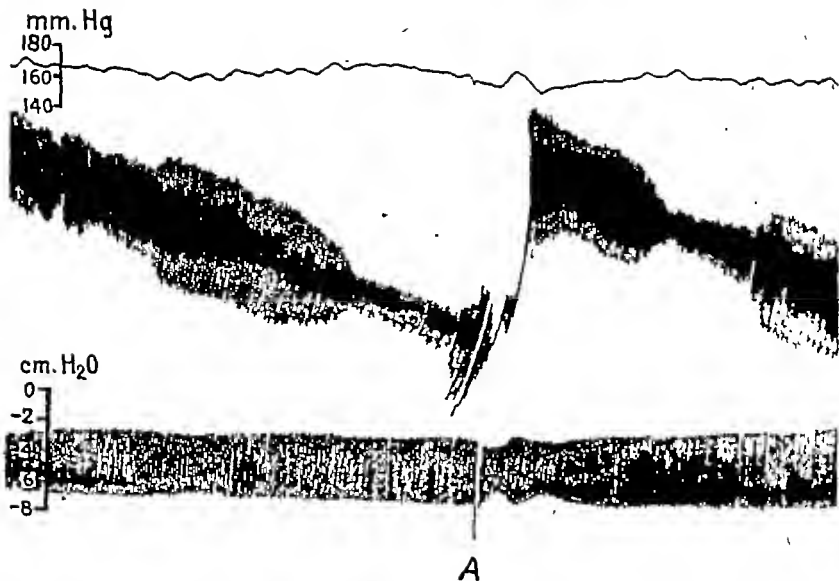
Fig. 2. Cat 51. 4·5 kg. Intraperitoneal dial 45 mg./kg. Tracings as in Fig. 1. Spontaneous periodic breathing without change in the pleural pressure fluctuation. At A, spirometer refilled.
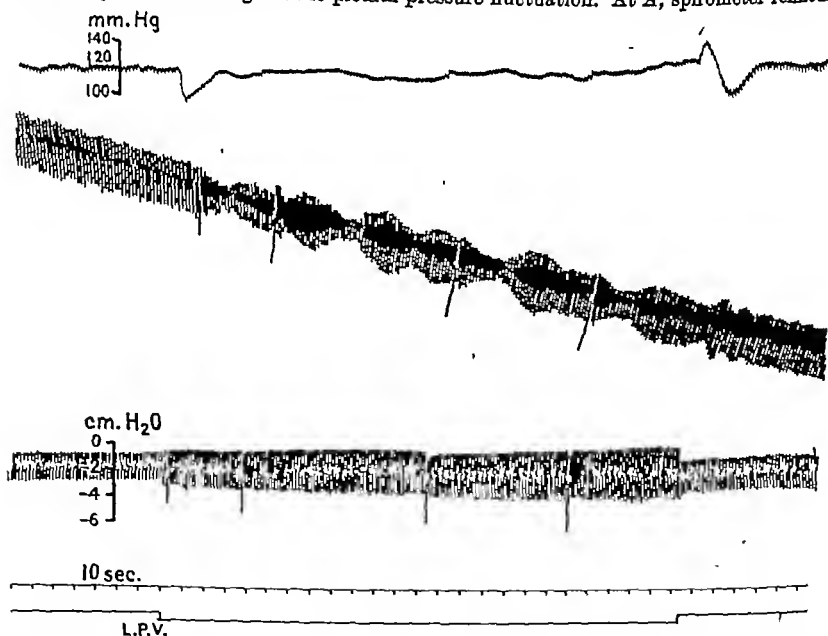
Fig. 3. Cat 50. 3·25 kg. Intraperitoneal dial 50 mg./kg. Tracings as in Fig. 1. Drinker heart preparation, with loose ligatures around the left pulmonary veins. Periodic breathing produced by occlusion of these veins ($L.P.V.$). Gradual slight increase in pleural pressure fluctuation. Normal breathing and return of pleural pressure fluctuations to their initial values when the pulmonary circulation was restored.

Fig. 3 is a tracing obtained from a Drinker heart preparation in the cat [Drinker, 1921]. An elliptical window was cut in the anterior chest wall over the heart, and the pleural cavities were closed by stitching the reflected pericardium to the margins of the window. The left pulmonary veins were exposed at their junction with the left auricle, and a ligature was put loosely around them so that gentle traction would cause occlusion of the veins. The effects of
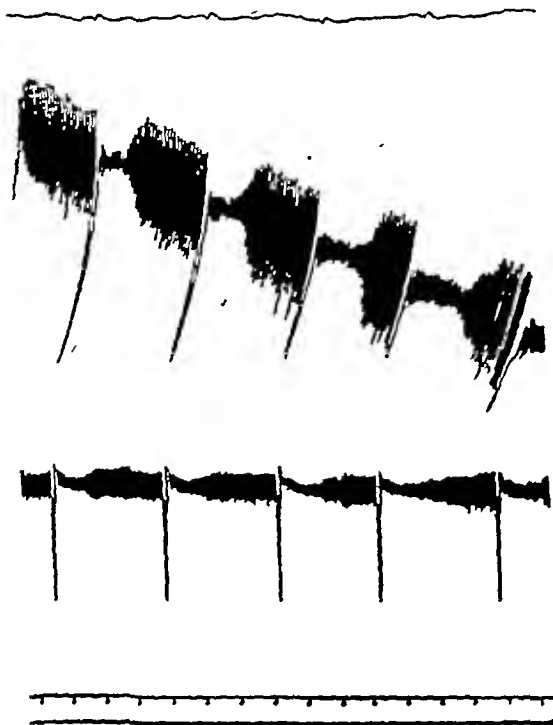
Fig. 4. Cat 58. 5 kg. Intraperitoneal dial 45 mg./kg. Tracings as in Fig. 1. Periodic breathing due to periodic changes in the activity of the respiratory centre. Changes in respiratory depth are associated with corresponding changes in pleural pressure.

occlusion of the pulmonary veins are shown in Fig. 3. After occlusion, a well-marked waxing and waning of the tidal air appears, without a corresponding change in the pleural pressure fluctuation, although both are seen to increase when the animal takes an occasional deep breath. There is a gradual slight increase in the pleural pressure fluctuation when the left pulmonary veins are occluded, but when the pulmonary circulation is restored breathing becomes regular again and the pleural pressure fluctuation returns to its initial value.

The contrast between this type of periodic breathing and the central type with altering activity of the respiratory centre is well shown in Fig. 4. Here the tidal air and pleural pressure vary together.

In all, fifteen animals (twelve cats and three rabbits) showing periodic breathing were studied in this investigation. Eleven showed the type of periodic breathing illustrated in Figs. 1–3. Four showed the type of breathing in Fig. 4. The periodic breathing arose spontaneously, either as soon as the animals were anaesthetized or, more often, at the end of some other experiment when the cardiovascular system was in poor condition.

DISCUSSION

These findings may be discussed (a) in terms of pulmonary dynamics, and (b) in their relationship to human periodic breathing (Cheyne-Stokes breathing). The type of periodic breathing described, with well-marked changes in tidal air and yet a constant fluctuation in pleural pressure, cannot be of central origin, since any changes in the intensity of the nervous discharges from the respiratory centre must be associated with increased respiratory effort and comparable changes in the pleural pressure readings. It must be due to some changes in the lung itself, whereby the same force exerted on the lung causes a varying amount of expansion. In other words, there is a periodic change in the distensibility of the lung. It appears almost certain that the cause of this change is a variation in the degree of pulmonary congestion. It is improbable that the change was due to broncho-spasm, as in six experiments adrenalin did not abolish the periodicity and there was no evidence of respiratory obstruction. Furthermore, the normal response to respiratory obstruction is an immediate increase in respiratory effort, and this did not occur [Christie, 1938].

It is known that the pulmonary vessels are supplied by sympathetic and parasympathetic nerves, and a periodic alteration in the discharges from the pulmonary vasomotor centre could, in this case, account for the respiratory changes. Fig. 1 is interesting in this respect, as Traube-Hering waves in the systemic circulation are associated with periodic breathing of the same frequency, suggesting that the same periodic alterations in the degree of vascular tone may take place in the pulmonary circuit.

Periodic variation in the tone of the pulmonary vessels has occasionally been noted in lung-perfusion experiments, but no observations on tidal air were made [Daly, Ludány, Todd & Verney, 1937]. In Fig. 3 periodic breathing was initiated each time one set of pulmonary veins was occluded, which also suggests that some alteration in the pulmonary circulation is responsible for the periodic breathing.

It has been established that the periodic breathing due to anoxaemia or depression of the respiratory centre differs in several respects from that seen in cardiovascular disease. In the former, the blood pressure rises during apnoea and falls during hyperpnoea, whereas in the latter exactly the reverse takes place [Eyster, 1906]. In cardiovascular disease, the duration of the cycle is usually much longer and there is a fluctuation in the respiratory level which
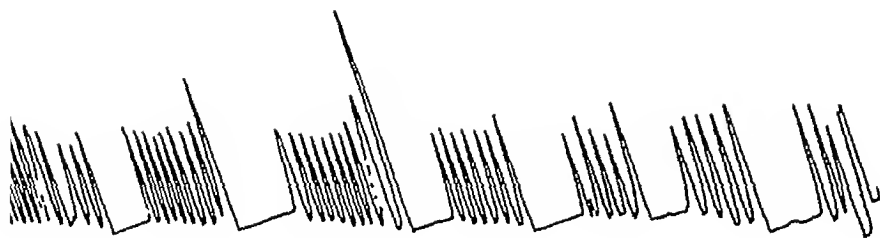
Fig. 5. Periodic breathing in a patient suffering from acute codeine poisoning. The patient was breathing pure oxygen from a spirometer and the tracing reads from right to left. Periodicity tends to diminish with oxygen and there is no fluctuation in respiratory level. Time signal, 1 min.

1000 c.c.
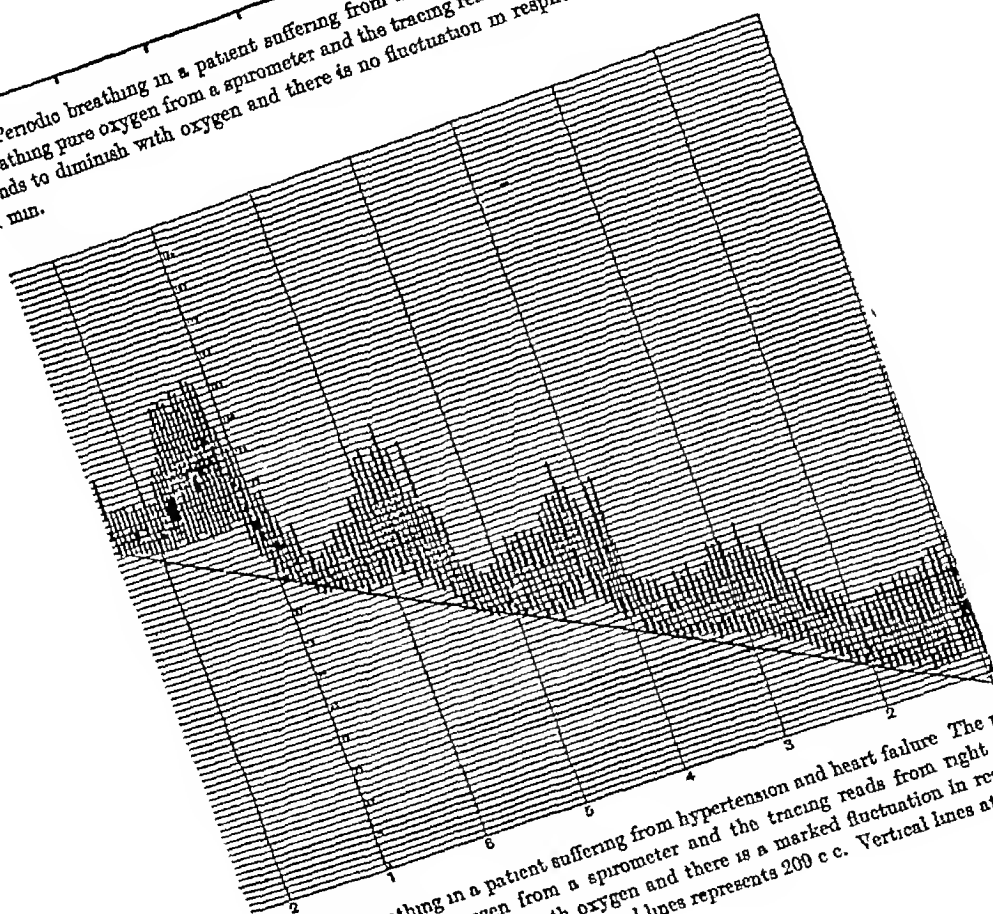
Fig. 6 Periodic breathing in a patient suffering from hypertension and heart failure The patient was breathing pure oxygen from a spirometer and the tracing reads from right to left. Periodicity tends to increase with oxygen and there is a marked fluctuation in respiratory level. Distance between heavy horizontal lines represents 200 c.c. Vertical lines at intervals of 1 min.

does not occur in Cheyne-Stokes breathing due to cerebral causes [Green, 1933] (Figs. 5 and 6). The response to oxygen therapy also serves to separate the two types. In the cerebral type oxygen usually abolishes Cheyne-Stokes breathing, whereas in the cardiovascular type oxygen usually produces either no change or may exaggerate the periodicity [Green, 1933] (Figs. 5 and 6).

These differences cast doubt on the usual physiological explanation of the mechanism of Cheyne-Stokes breathing, based almost entirely on experiments with anoxia. The mechanism of Cheyne-Stokes breathing in cardiovascular disease may be fundamentally different, and it seems possible that the fluctuations in respiratory depth associated with an alteration in respiratory level may be due, in part at least, to a periodic alteration in the degree of pulmonary congestion.

There is thus a possible relationship between the types of periodic breathing we have described in animals, and which we interpret as being caused by a waxing and waning pulmonary congestion, and the type of periodic breathing seen clinically in patients with cardiovascular disease. There are certain differences, however, which must be noted. The alterations in respiratory level which are characteristic of spirometer tracings in Cheyne-Stokes breathing in the cardiovascular group have not been shown in any of the animals. In cardiovascular disease, the administration of 5 % CO_2 in oxygen, or 'euphyllin' (theophyllin and ethylenediamine) intravenously, usually abolishes Cheyne-Stokes breathing, whereas, in these animals, carbon dioxide merely increased the hyperpnoea, and 'euphyllin' (theophyllin and ethylenediamine) was without effect.

SUMMARY

Periodic changes in respiratory depth may occur in anaesthetized animals, which are due to changes in the distensibility of the lung and not to any change in the activity of the respiratory centre.

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THE MECHANISM OF THE ·VASOMOTOR REFLEXES PRODUCED BY STIMULATING MAMMALIAN SENSORY NERVES

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It has often been suggested that afferent impulses, in response to a nocuous stimulus, have a part in initiating the changes in blood pressure which may follow severe injury. The idea gets an added interest from some recent suggestions that these impulses are carried in at least two types of fibre, the delta fibre and the unmyelinated C fibre [Zotterman, 1939], and it calls for a close analysis of the reflex changes in blood pressure produced by various kinds of electrical stimulation of an afferent nerve.

It is known that stimulation of the central end of the cut sciatic nerve in the cat can have either a pressor or a depressor effect. Weak stimulation [Hunt, 1895], or stimulation at low frequencies [Gruber, 1917], gives a depressor reflex; if either the strength or frequency is increased, the reflex may become pressor. Two views are held about this reversal, produced by increasing either strength or frequency of stimulation; they are reviewed by Ranson [1921], who believed that the pressor reflex was related to a greater central summation of impulses. Hunt [1895], on the other hand, had suggested that the pressor and depressor reflexes were served by different types of afferent fibre, the pressor fibre having a higher threshold.

This question is reopened here. Clark, Hughes & Gasser [1935] found that there is still a vasomotor reflex if all the fibres in the sensory nerve except the unmyelinated C fibres have been inactivated by asphyxiating the nerve. Their experiment has been repeated here under controlled conditions, to find the nature of this vasomotor reflex; and dissociation of fibres has also been produced here by cocaine, which affects the smallest fibres first [Gasser, 1935], and by cooling, which produces a sensory dissociation that is not closely related to fibre diameter [Bickford, 1939].

METHODS

Cats were used for the experiments. Thirty out of thirty-six were anaesthetized with nembutal, given intraperitoneally in the dose of 35 mg./kg. body weight. Deep anaesthesia was maintained by giving 10–20 mg. intravenously at intervals. Three animals were anaesthetized with urethane,

given by stomach tube in the dose of 2 g./kg. body weight: the stomach tube was passed under ethyl chloride anaesthesia. Three other animals were decerebrated under ether, and were subsequently given 10 mg./kg. of curare intravenously, and then ventilated artificially. In the anaesthetized animals, care was taken to maintain a deep and as far as possible a constant level, so as to prevent any visible reflex movements during stimulation or any vigorous respiratory reflexes, since either of these would give rise to uncontrolled secondary changes in the blood pressure.

The nerve usually chosen for stimulation was the medial popliteal, and its extension, the posterior tibial. The nerve was dissected out from the middle of the thigh down to the ankle, without cutting the arteries supplying it, and all its muscular branches in this region were cut. The nerve itself was usually cut through at the ankle. The electrodes were of platinum, housed in perspex holders of the Sherrington type.
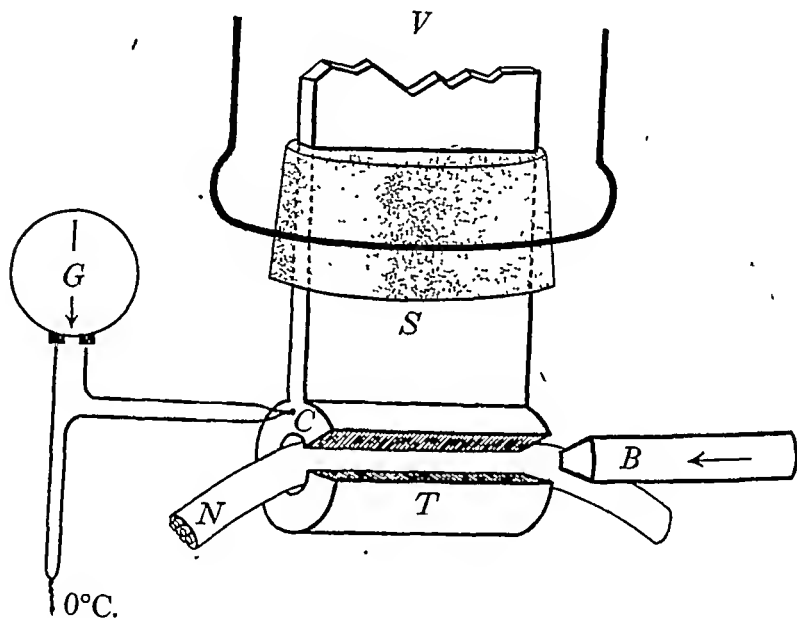
Fig. 1. Diagram of the apparatus used for cooling or freezing nerves. For description see text.

Stimulation was provided by condenser discharges. Stimuli with time constants of either 1 or 0·1 msec. were used; the former were provided by a neon tube stimulator of conventional design, and the latter by a hard valve circuit. These instruments were calibrated for strength, frequency, and duration of stimulus on a cathode-ray oscilloscope. The time constant (RC) of a condenser discharge in sec. was calculated from the stimulator circuit: it is the product of the capacity of the discharging condenser in μF. and the resistance through which it discharges in MΩ.

Asphyxia of a nerve was produced either by a sphygmomanometer cuff tied round the limb and inflated to 40 mm. above arterial blood pressure, or by tying the arteries supplying blood to the limb. It was found that a number of arteries must be tied if the blood supply is to be cut off completely; otherwise a collateral circulation is soon established. Permanent ligatures were tied round the last two pairs of lumbar arteries, both internal iliac arteries, the caudal artery, and both inferior epigastric arteries. A loose loop of silk was then passed round the external iliac artery of the side to be asphyxiated later on, and this was brought out of the abdominal incision through a short flanged ebonite tube, round which the skin and muscles were stitched up. When it was necessary to asphyxiate the limb, the artery was lifted up by this loop, and was occluded with a rubber-padded clip passed down the tube. The efficiency of this method was tested by injecting

1% trypan blue into the jugular vein with the arterial ligatures in position, and if there was no colouring of the limb in question after 15 min., it was considered that its blood supply had been cut off.

Nerves were *cooled* with the apparatus shown in Fig. 1. The cooling element itself is a copper tube (*T*), with a longitudinal slot cut in it, through which the nerve (*N*) is inserted. The tube is ¼ in. long, and its wall is about three-quarters of the thickness of the nerve it contains. A block of copper (*B*) slides into the slot, and completes the encirclement of the nerve. Both this block and the tube are silver-plated. The tube is soldered to a vertical copper strip (*S*) ½ in. thick, which passes up through a rubber bung into a vacuum flask (*V*), containing either ice or an ice and salt mixture. The temperature of the tube *T* is measured from a thermocouple soldered into it (*C*), and lying as close to the nerve as possible. Another thermocouple is kept at $0°$ C. A range of temperatures down to $-6°$ C. can be obtained.

RESULTS

Stimulation of normal nerves

Relation of the reflex to the strength of stimulation. Fig. 2 illustrates an experiment in which the central end of the posterior tibial nerve was stimulated with shocks of time constant 1 msec. and frequency 3 per sec. The nerve was stimulated for periods of 20 sec., starting with a strength of 4 V., and increasing in subsequent stimulations up to 28 V. The results are typical of all the experiments in the series. There is a fall of blood pressure with weak stimuli of 4, 6, and 12 V., a fall followed by a rise with an intermediate stimulus of 22 V., and a rise with the strongest stimulus of 28 V.

The accompanying record of respiration shows a slight decrease in amplitude when the blood pressure fell, and an increase when the blood pressure rose.

. *Relation to the duration of the stimulus.* When a stimulus was used with the shorter time constant of 0·1 msec., it was found impossible to produce any pressor reflexes, even with strong stimuli (60–80 V.), provided that the frequency of stimulation was less than 100 per sec. This is illustrated by Fig. 3, which contrasts the depressor reflex, obtained at 60 V. with a short-lasting stimulus, with the large pressor reflex obtained at 25 V. with a longer stimulus. The stimulus frequency was kept constant at 10 per sec.

Relation to the frequency of stimulation. If the strength of stimulation is such that depressor reflexes are obtained with a low stimulus frequency, pressor reflexes can often be obtained by increasing the frequency of stimulation alone (Fig. 4). This reversal usually happens when the frequency is raised above 100 per sec., and it was found even with a stimulus of short time constant (0·1 msec.), though the reflex rise in blood pressure was then never more than 20 mm. of mercury.

All the reflex effects described so far have been found consistently in animals anaesthetized with nembutal or urethane, and in curarized decerebrated animals. The type of reflex response was not affected by cutting both vagi and cauterizing the tissues round the carotid sinus, although both pressor and depressor reflexes were larger after this had been done.
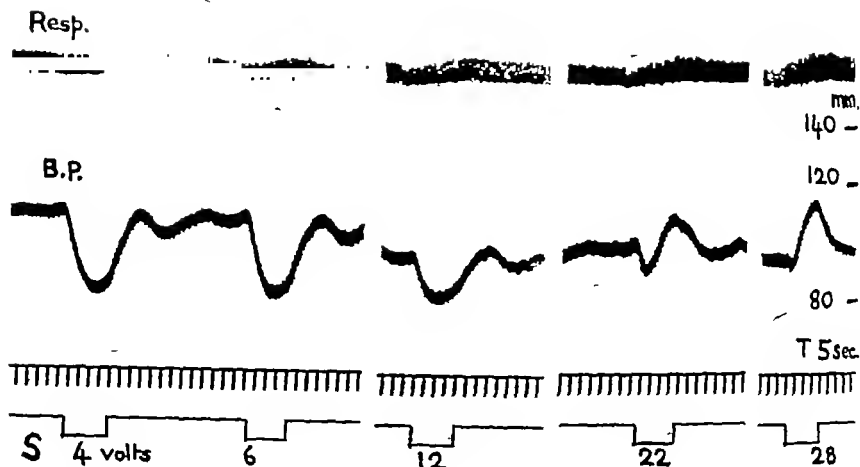
Fig. 2. Records of the vasomotor (*B.P.*) and respiratory (*Resp.*) reflexes produced by stimuli of various strengths applied to the central end of the posterior tibial nerve. Weak stimulation gave a depressor reflex and strong stimulation a pressor reflex. Respiration recorded by Gaddum's method. Anaesthetic, urethane. Frequency of stimulation, 3 per sec. Time constant, 1 msec.
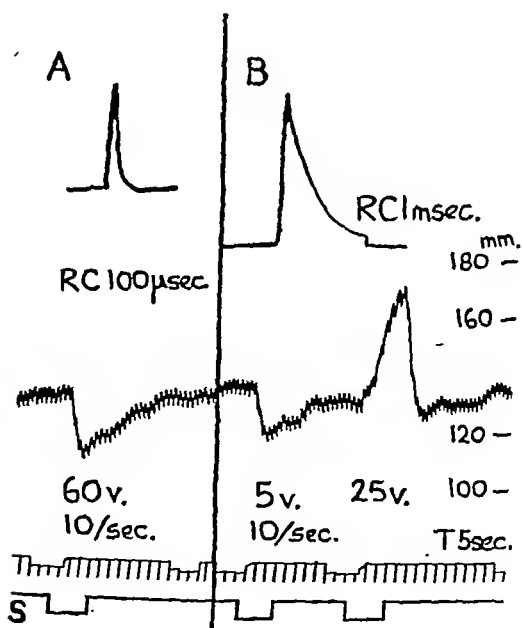
Fig. 3. Records of the reflex changes in blood pressure produced by stimulating the central end of the posterior tibial nerve. In *A* a strong stimulus of short time constant (0·1 msec.) gave a depressor reflex. In *B* the time constant was 1 msec. and a pressor reflex was obtained with the weaker stimulus of 25 V. Anaesthetic, nembutal. Frequency of stimulation 10 per sec. The tracings inset above show the configuration of the appropriate stimuli: they were taken from cathode-ray oscilloscope records.

In the remaining experiments described below, a stimulus with a time constant of 1 msec. has been used, and the frequency has usually been kept constant throughout each experiment. Pressor and depressor reflexes have been produced by strong and weak stimulation respectively, and the nerve has been altered, proximal to the stimulated point, by cocainizing, asphyxiating,
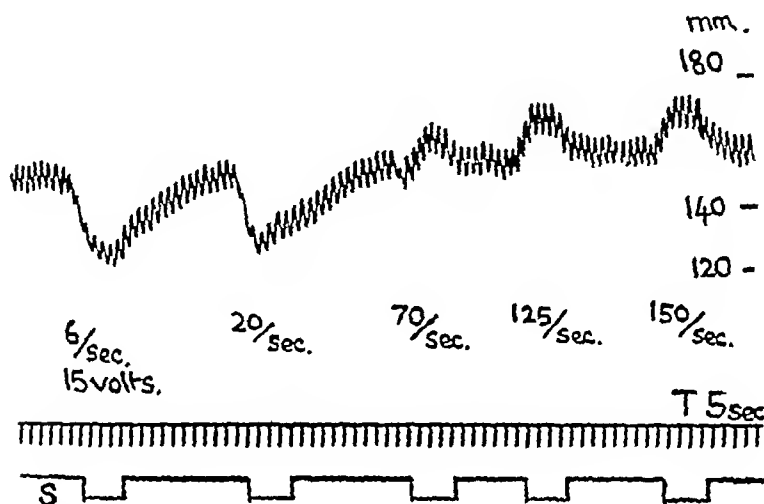
Fig. 4. Records of the reflex changes in blood pressure produced by stimulating the central end of the posterior tibial nerve at a fixed strength of stimulus but with a variable frequency. Anaesthetic, nembutal. Strength of stimulus, 15 V. Time constant, 1 msec.

or cooling. Possible errors caused by the spread of electrical stimulus across the block have been eliminated by placing the distal electrodes at least 1 in. beyond the block, and, in most experiments, by using a control pair of electrodes proximal to the block.

The effect of cocaine

Two pairs of electrodes were used, one applied to the posterior tibial nerve at the ankle, and the other to the same nerve above the knee. Before applying cocaine, control stimulation was carried out at each pair of electrodes with weak and strong stimuli. 0·5 c.c. of 1 % cocaine hydrochloride was then slowly infiltrated under the nerve sheath between the pairs of electrodes, through a very fine hypodermic needle. After this the nerve was stimulated at each pair of electrodes every minute, and the vasomotor reflexes obtained by stimulation proximal and distal to the narcotized portion were compared.

Five minutes after applying the cocaine, no pressor reflex could be obtained by stimulating distally (Fig. 5), and a depressor reflex was the response

to the strongest stimulation (30 V.). The reflexes obtained by stimulating proximally were unchanged. After 15 min. the nerve was usually completely blocked by the cocaine, and reflexes were only obtained by stimulating proximally.
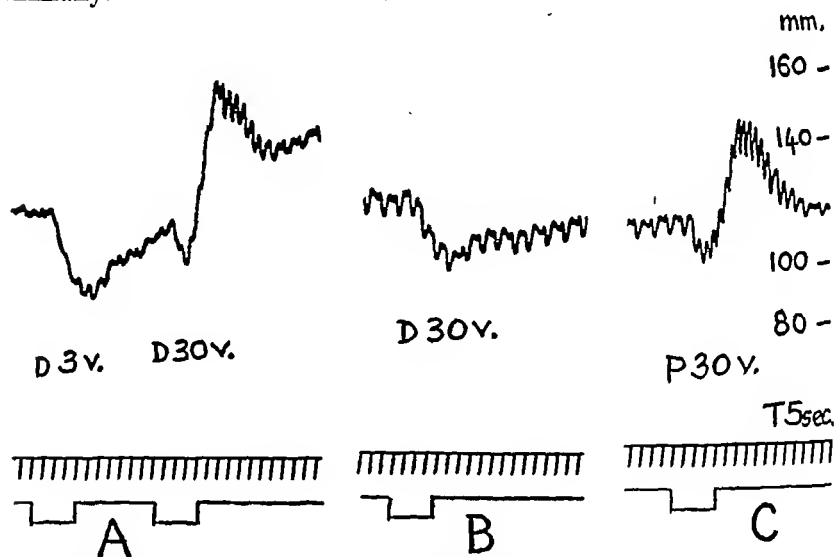
Fig. 5. Records showing the effect on the vasomotor reflexes of applying cocaine to the nerve proximal to the stimulated point. A. Depressor and pressor reflexes obtained with weak and strong stimuli before applying cocaine. B. Depressor reflex obtained by strong stimulation distally, 5 min. after applying 0·5 c.c. 1 % cocaine. C. Pressor reflex obtained by strong stimulation proximal to the cocainized point. Anaesthetic, nembutal. Frequency of stimulation, 2 per sec. Time constant, 1 msec.

The effect of asphyxia

Electrodes were applied to the posterior tibial nerve of each leg. It was confirmed before starting the experiment that the vasomotor reflexes obtained by stimulating each nerve were similar for the same strengths of stimulus. One leg was then asphyxiated, either with a sphygmomanometer cuff or by tying the arteries supplying it.

There was no change in the reflexes from the asphyxiated side for 30–40 min., but after this the depressor reflex obtained with weak stimulation became smaller, and finally disappeared after 50–60 min. At this stage of asphyxia it was still possible to obtain pressor reflexes with strong stimulation (30 V.), and often small pressor reflexes were seen with the weaker stimuli, which had given only depressor reflexes before asphyxia. The pressor reflex response to strong stimulation (30 V.) of the asphyxiated nerve was usually slightly larger than that obtained when the same stimulus was applied to the normal nerve of the other leg.

When the blood supply was restored, the depressor reflex appeared again, and after 10 min. there was no difference between the reflexes obtained by stimulating the two nerves (Fig. 6).
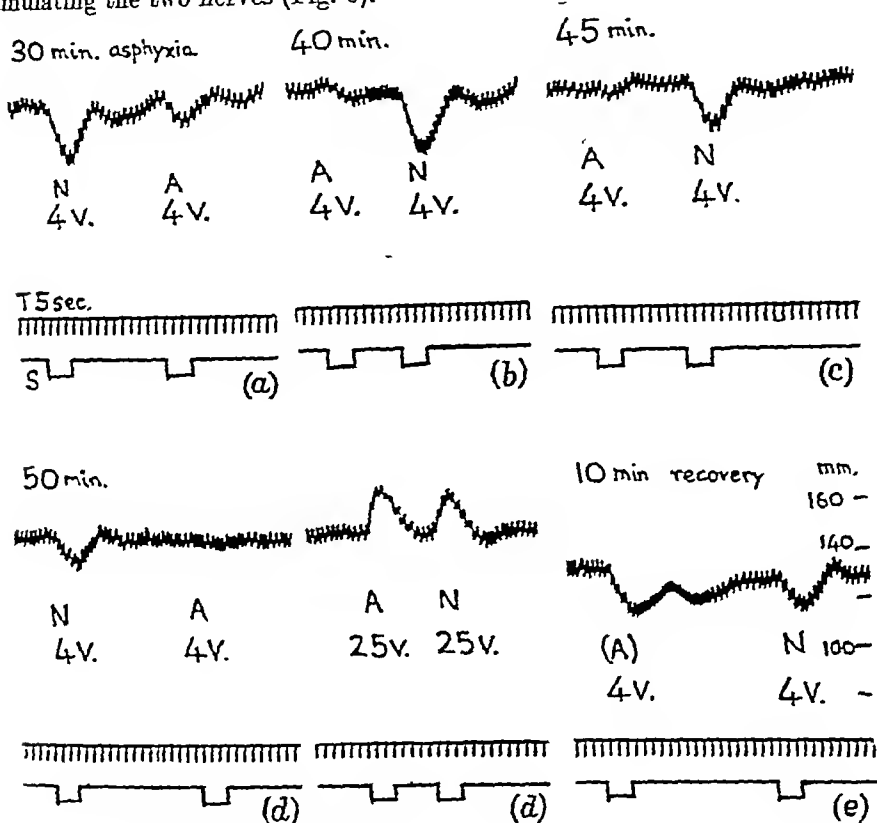
Fig. 6. Records of the vasomotor reflexes produced by stimulating the posterior tibial nerves. One nerve (A) had been asphyxiated by tying the arteries supplying the appropriate limb; the other nerve (N) was normal. (a) Weak stimulation of each nerve (4 V.) 30 min. after occlusion of the blood supply. (b) Similar stimulation after 40 min. (c) Similar stimulation after 45 min. (d) Weak (4 V.) and strong (25 V.) stimulation of each nerve after 50 min. (e) Weak (4 V.) stimulation of each nerve 10 min. after removing the arterial clip and restoring the blood supply. The depressor reflex from the asphyxiated nerve is abolished after 50 min., though the pressor reflex remains at the end of this time. The depressor reflex appears again after restoring the blood supply. Anaesthetic, nembutal. Frequency of stimulation, 3 per sec. Time constant, 1 msec.

Asphyxia, therefore, in contrast to cocaine, abolishes the depressor reflex first, but the pressor reflex remains, and is often rather larger than before.

The effect of cooling and freezing

Cooling. The nerve was cooled to various temperatures between 4 and 10° C. with the apparatus which has been described (Fig. 1). It was stimulated distal

to the cooled point, after 10 min. had been allowed for the nerve to reach an
even temperature. After stimulation, the cooling element was removed, and
stimulation was repeated after 5–15 min., when the nerve had returned to body
temperature.

When the nerve was cooled to temperatures between 5 and 7° C., the de-
pressor reflex obtained with weak stimulation at 2 per sec. was smaller than
normal, and the pressor reflex with strong stimulation was sometimes unaltered
and sometimes slightly larger than normal.

When the nerve was cooled to 4° C., no depressor reflex could be obtained at
all, and the pressor reflex was rather smaller than normal (Fig. 7). When the
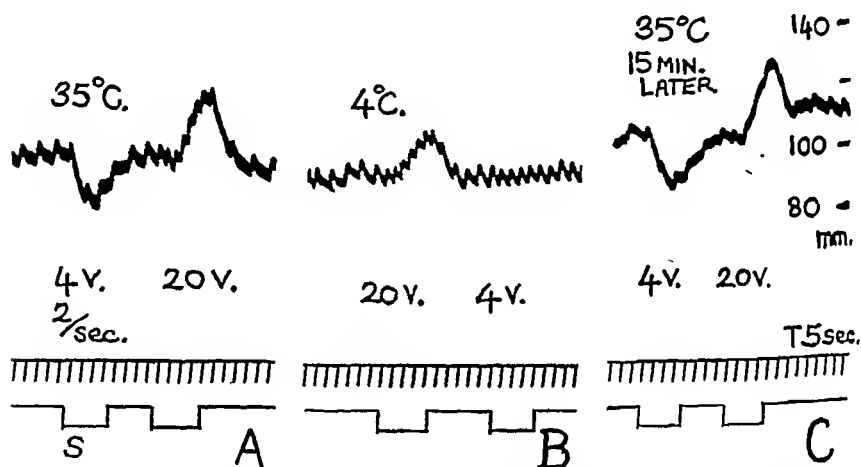
Fig. 7. Records showing the effect on the vasomotor reflexes of cooling the nerve to 4° C. proximal
to the stimulated point. A. Depressor and pressor reflexes obtained with weak and strong
stimuli before cooling. B. Depressor reflex abolished but pressor reflex still present after
cooling to 4° C. C. Depressor and pressor reflexes obtained as before, 15 min. after removing
the cooling element. Anaesthetic, nembutal. Frequency of stimulation, 2 per sec. Time
constant, 1 msec.

cooling element was removed, both pressor and depressor reflexes reappeared
within a few minutes.

Cooling the nerve to temperatures below 4° C. abolished both pressor and
depressor reflexes.

Freezing. Two pairs of electrodes were applied to the nerve, and the cooling
element between them. The nerve was cooled to −3 or −4° C. with an ice and
salt mixture, and kept at that temperature for 5–7 min. By the end of that
time it was frozen hard, and it was removed from the cooling tube with a
camel-hair brush moistened with warm Ringer-Locke solution.

Stimulation at each pair of electrodes was then carried out every 5 min.
There was usually no vasomotor reflex response to stimulation at the distal pair
of electrodes for about 10 min. A pressor reflex then appeared on strong

stimulation, and as stimulation was repeated this gradually became larger, until after an hour or more the strongest stimulation (30 V.) gave a much larger
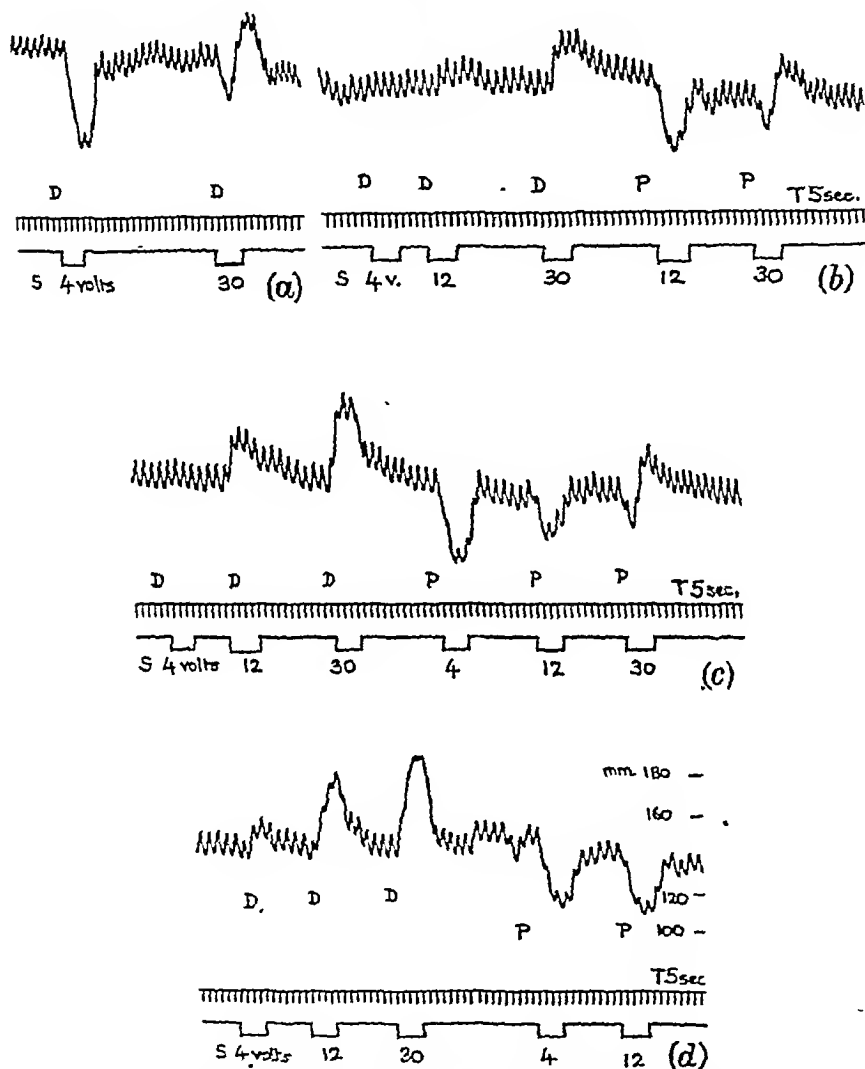
Fig. 8. Records showing the effects on the vasomotor reflexes of freezing the nerve (7 min. at $-4°$ C.). Two pairs of electrodes were used, proximal (P) and distal (D) to the point frozen. (a) Depressor and pressor reflexes obtained with weak and strong stimulation before freezing. (b) Stimulation at distal and proximal electrodes 20 min. after freezing.   (c) Stimulation after 40 min.   (d) Stimulation after 80 min. The pressor reflex returns after freezing; it is larger than before and can be obtained with weaker stimuli. The depressor reflex does not return. Anaesthetic nembutal. Frequency of stimulation, 6 per sec. Time constant, 1 msec.

pressor reflex than could be obtained by stimulating at the proximal pair of electrodes. At this stage, a weak stimulus (4 V.) also gave a small pressor

reflex, though the same weak stimulus at the proximal electrodes gave a large depressor reflex (Fig. 8).

It was usually impossible to obtain depressor reflexes by stimulating distal to the portion of nerve which had been frozen, at any time during the rest of the experiment, but sometimes there was a partial recovery after 2 hr. or more, and then a small depressor reflex was obtained again.

The effect of cooling or freezing the sensory nerve resembles that of asphyxia in that the depressor reflex is the first to be abolished by cooling, and the last to return after freezing.

DISCUSSION

These experiments confirm the previous observations that the type of vaso-motor reflex obtained by stimulating the central end of a cut sensory nerve depends on both the strength of stimulation [Hunt, 1895], and the stimulus frequency [Gruber, 1917]. They also emphasize the importance of a sufficiently long shock-duration in obtaining pressor reflexes. Ashkenaz [1939] claimed that it was not possible to produce a reversal from a depressor to a pressor reflex merely by increasing the strength of stimulation; but his results suggest that he used shocks of too short a duration (see Fig. 3).

The experiments also throw some light on the question originally discussed by Ranson & Billingsley [1916], who suggested that any influence which re-duced the number of impulses in an afferent nerve would tend to abolish the pressor reflex, and that it would then be easier to obtain a depressor reflex. They believed, therefore, that the appearance of a pressor reflex was the result of increased central summation. The results described above show that nerves blocked by cocaine fulfil this prediction, but that the opposite is true of blocks produced by asphyxia or cold. There can be no doubt that each of these three blocking agents reduces the number of nervous impulses passing centrally; the abolition of the depressor reflex and the persistence of the pressor reflex after asphyxia or cooling cannot therefore be explained on the assumption that the pressor reflex is simply the expression of increased central summation.

These results, on the other hand, support the classical idea that sensory nerves contain pressor and depressor afferent fibres of different sizes, the pressor fibres having a higher threshold to stimulation, and being the more resistant to asphyxia or cooling, while the depressor fibres have the lower threshold, and are more resistant to cocaine. If two types of afferent peripheral fibre do exist, it is not unreasonable that the pressor reflex is larger than normal when the depressor antagonist has been inactivated by asphyxia, cooling, or freezing.

No attempt has been made here to identify the precise types of fibre re-sponsible for the pressor and depressor reflexes by means of their action poten-tials, but some tentative conclusions can be made about them. Clark et al. [1935] found from the action potential that the only fibres resisting asphyxia

for 45 min. were unmyelinated C fibres, and it has been shown here that the only vasomotor reflex persisting after the sensory nerve had been asphyxiated for this time was a pressor reflex. It is probable, therefore, that many of the pressor afferent fibres belong to the C group. This means that they have a higher threshold than the depressor fibres, and a longer excitation-time (Fig. 3).

The depressor afferent fibres, on this supposition, must belong mainly to the A group, being those which are the most resistant to narcosis with cocaine, and the least resistant to asphyxia. It is impossible, without the electrical evidence of the action potential, to tell to which subdivision of the A group these fibres belong. '

The effect of cooling and freezing the sensory nerve, in these experiments, has been similar to that of asphyxiating it, and although the effects of cold on the different types of fibre are not accurately known, it is significant that human 'second pain', which is probably mediated by unmyelinated C fibres [Lewis & Pochin, 1938], is the last sensation except warmth to be lost when a cutaneous nerve is cooled [Bickford, 1939]. Some further experiments are in progress on the effects of cold on the different fibres of a peripheral nerve.

There is one discrepancy between the results given here and those described by Hunt [1895]. Hunt found that cooling a sensory nerve first abolished the pressor reflex obtained by stimulating the nerve, and that the depressor reflex persisted longer. The entirely opposite result described here may depend on the fact that a stimulus of 2 per sec. frequency was used, while Hunt used a repetitive stimulus from an inductorium, with presumably a higher frequency. The use of a higher frequency of stimulation, when the nerve is at a low temperature, must bring in the complication of Wedensky inhibition, and it is probable that the smallest fibres, by virtue of their long refractory period, are more susceptible to this inhibition than larger fibres. In confirmation of this idea, it has occasionally been observed here that when a nerve was cooled to about 6° C., a reversal from a pressor to a depressor reflex was produced by increasing the frequency of stimulation from 2 to 20 per sec. This effect was only seen when the nerve was at a low temperature at the time of stimulation: it did not occur in nerves partially blocked by freezing, which had returned to body temperature before stimulation.

The reversal from a depressor to a pressor reflex, which occurred when the frequency of stimulation was raised above 100 per sec. (Fig. 4), and which Gruber [1917] was the first to observe, may depend on an increase in central summation of the kind which Ranson & Billingsley [1916] had proposed, and which has recently been discussed by Ashkenaz [1939]. It is a phenomenon of a different kind from the reversal produced by increasing the strength of stimulation; the latter is more likely to depend on the higher threshold of the pressor fibre.

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The depressor afferent fibres, on this supposition, must belong mainly to the A group, being those which are the most resistant to narcosis with cocaine, and the least resistant to asphyxia. It is impossible, without the electrical evidence of the action potential, to tell to which subdivision of the A group these fibres belong. '

The effect of cooling and freezing the sensory nerve, in these experiments, has been similar to that of asphyxiating it, and although the effects of cold on the different types of fibre are not accurately known, it is significant that human 'second pain', which is probably mediated by unmyelinated C fibres [Lewis & Pochin, 1938], is the last sensation except warmth to be lost when a cutaneous nerve is cooled [Bickford, 1939]. Some further experiments are in progress on the effects of cold on the different fibres of a peripheral nerve.

There is one discrepancy between the results given here and those described by Hunt [1895]. Hunt found that cooling a sensory nerve first abolished the pressor reflex obtained by stimulating the nerve, and that the depressor reflex persisted longer. The entirely opposite result described here may depend on the fact that a stimulus of 2 per sec. frequency was used, while Hunt used a repetitive stimulus from an inductorium, with presumably a higher frequency. The use of a higher frequency of stimulation, when the nerve is at a low temperature, must bring in the complication of Wedensky inhibition, and it is probable that the smallest fibres, by virtue of their long refractory period, are more susceptible to this inhibition than larger fibres. In confirmation of this idea, it has occasionally been observed here that when a nerve was cooled to about 6° C., a reversal from a pressor to a depressor reflex was produced by increasing the frequency of stimulation from 2 to 20 per sec. This effect was only seen when the nerve was at a low temperature at the time of stimulation: it did not occur in nerves partially blocked by freezing, which had returned to body temperature before stimulation.

The reversal from a depressor to a pressor reflex, which occurred when the frequency of stimulation was raised above 100 per sec. (Fig. 4), and which Gruber [1917] was the first to observe, may depend on an increase in central summation of the kind which Ranson & Billingsley [1916] had proposed, and which has recently been discussed by Ashkenaz [1939]. It is a phenomenon of a different kind from the reversal produced by increasing the strength of stimulation; the latter is more likely to depend on the higher threshold of the pressor fibre.

It remains to discuss the possible importance of these principles in the clinical question of the vasomotor reactions after severe injury. C fibres, on the evidence of experiments on human beings [Lewis & Pochin, 1938] and on animals [Zotterman, 1939], carry slowly conducted impulses associated with 'second pain'. These fibres adapt slowly, and their discharge, in response to a single cutaneous stimulus in the cat, is apt to be prolonged. Most of the pressor afferent fibres probably belong to this group. On the evidence of these same authors, the fibres carrying impulses of the accurately localized 'first pain' belong to the A group, and are probably delta fibres. This group probably contains depressor rather than pressor afferents; and it may be that afferent impulses in these fibres, which adapt more rapidly, can play a part in the reaction to injury which is known as primary shock, and from which recovery is often spontaneous. But if any nervous discharge has to do with the more serious condition of secondary traumatic shock, a question which remains very controversial, it is more likely to be a discharge in slowly adapting fibres, possibly in those of the C group, which appears to have given a pressor reflex under the experimental conditions described in this paper.

SUMMARY

1. Electrical stimulation has been applied to the central end of the cut posterior tibial and other mixed nerves in cats, which were either deeply anaesthetized with nembutal or urethane, or else decerebrated. Stimulation at low frequencies causes a reflex rise in blood pressure with strong stimuli, and a fall with weak stimuli. This agrees with the results obtained by Hunt [1895]. Only depressor reflexes can be produced, however, even with very strong stimulation, when the time constant of the stimulus is as short as 0·1 msec.

2. When cocaine, an agent which inactivates the smallest fibres first [Gasser, 1935], is applied to the nerve proximal to the point stimulated, the pressor reflex is abolished before the depressor reflex.

3. When the nerve is blocked by cold or asphyxia, the depressor reflex is abolished first, and the persisting pressor reflex often becomes larger than before. Asphyxia, in contrast to cocaine, affects the largest fibres first [Clark et al. 1935]: the effect of cold on fibres of different sizes is not so clearly known.

4. On this evidence, it is suggested that the pressor and depressor reflexes have separate afferent fibres in sensory nerves, the pressor fibres possibly belonging to the C group, and having a higher threshold to stimulation.

5. If the strength of stimulation is kept constant, a reversal from a depressor to a pressor reflex can be produced by increasing the frequency of stimulation alone, as Gruber [1917] originally found. It is suggested that this type of reversal depends on central summation of impulses.

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EFFECTS OF IODOACETIC ACID, GLYCERALDEHYDE AND PHOSPHORYLATED COMPOUNDS ON THE SMALL INTESTINE OF THE RABBIT

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The stimulating action, on the longitudinal muscle of the rabbit's intestine, of glucose and pyruvate, results mainly from their ability to supply the chemical energy necessary for the normal tonic and rhythmic activity of this muscle [Feldberg & Solandt, 1942]. The question thus presented itself how substances like iodoacetic acid and glyceraldehyde, which influence carbohydrate metabolism, would effect the stimulating action of glucose and pyruvate, and further, if phosphorylated carbohydrates or their derivatives were able to replace glucose or pyruvate in their stimulating action on the smooth-muscle preparation of the intestine.

According to Prasad [1935] the longitudinal muscle of the intestinal preparation poisoned by iodoacetic acid is no longer stimulated by glucose, whereas pyruvate has a definite but slight beneficial effect. Glyceraldehyde was found to inhibit glycolysis in tissue slices or extracts of tumours [Mendel, 1929], of brain [Holmes, 1934; Ashford, 1934] and of embryo [Needham & Nowinski, 1937; Needham & Lehmann, 1937]. Reversal of the glyceraldehyde inhibition by pyruvate has been observed in tumour tissue [Mendel, Bauch & Strelitz, 1931] and embryo brei [Needham & Nowinski, 1937]. According to Mann, Tennenbaum & Quastel [1938], glyceraldehyde inhibits acetylcholine formation by minced brain tissue in the presence of glucose. Kahlson & MacIntosh [1939] obtained no definite effect of this kind in their experiments on perfused superior cervical ganglia. No experiments have hitherto been carried out to demonstrate the inhibitory action of glyceraldehyde on surviving smooth-muscle preparations.

METHODS

A piece of 6–7 cm. from the duodenum or upper third of the small intestine of the rabbit was suspended in a 14 c.c. bath. The lumen of the intestine was left open at both ends, the oral end being attached to a thread leading to a Lovatt Evans frontal writing lever. Tyrode solution, with and without glucose, was

used, air bubbled through it and the temperature kept between 33 and 36° C. The bath was emptied by overflow and the substances were added with a syringe. The phosphoric esters were used as sodium salts.

RESULTS

Iodoacetic acid

Iodoacetic acid inhibits the stimulating action of glucose. Therefore, when added, in a concentration of 1 : 2000–5000, to a preparation suspended in aerated Tyrode solution containing glucose, the longitudinal muscle relaxes and
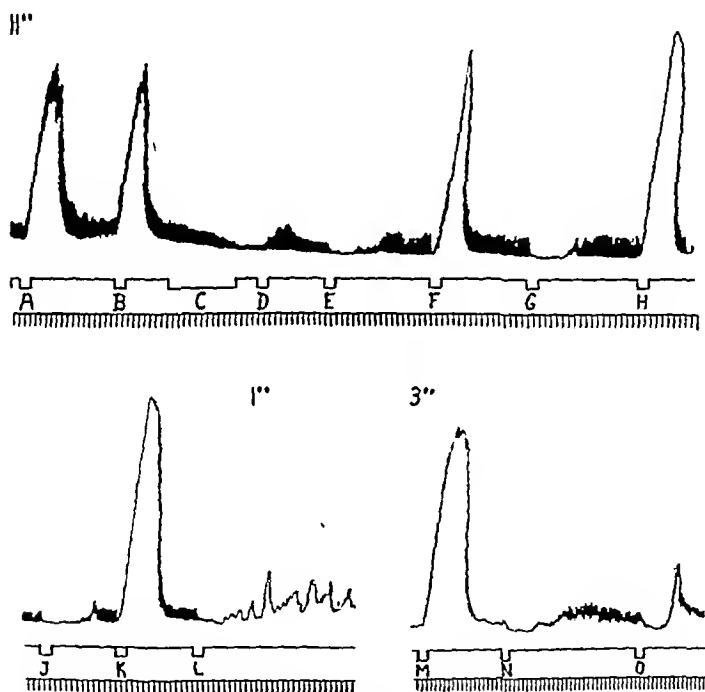
Fig. 1. Record of rabbit's small intestine preparation in 14 c.c. glucose-free Tyrode solution. Effect of 2 mg. iodoacetic acid (at C) on 10 mg. glucose (at A, E, G, J, L, N and O) and on 0·5 mg. sodium pyruvate (at B, D, F, H, K and M). Time in 30 sec. For details see text.

the amplitudes of the rhythmic contractions diminish. This inhibition, which may be preceded by a slight contraction, starts after a latency of a minute or two and increases gradually even if the iodoacetic acid is washed out after a few minutes. When added to a preparation suspended in glucose-free Tyrode solution it has no action of this kind or causes slight diminution in the size of the rhythmic contractions only (Fig. 1 C). In this condition, however, it renders the muscle inexcitable to the stimulating action of glucose, an effect which may last for hours after the iodoacetic acid has been washed out. The

stimulating action of sodium pyruvate is also inhibited, but to a lesser degree and for a shorter period. It is thus possible to obtain a condition in which the muscle no longer responds to the stimulating action of glucose but still to that of pyruvate. This is illustrated in Fig. 1. The preparation was suspended on 14 c.c. glucose-free Tyrode solution; an hour later when the longitudinal muscle was well relaxed and the rhythmic contractions had become small glucose and pyruvate produced strong contractions, 10 mg. of glucose (at A) being about as active as 0·5 mg. of sodium pyruvate (at B). With pyruvate the latency (13 sec.) was shorter than with glucose (23 sec.), and the contraction reached its maximum earlier and subsided more quickly after replacing the bath with glucose-free Tyrode solution. At C, 2 mg. of iodoacetic acid were added to the bath and washed out after 6 min. One minute later, at D, pyruvate no longer stimulated the muscle, but after 18 min., at F, it stimulated the muscle nearly as strongly as at B. Some inhibition was revealed by the long latent period of 59 sec. The latency at H was 36, at K 26 and at M 29 sec. In other experiments lengthening of the latent period persisted for some time only after the iodoacetic acid had been washed out. The stimulating action of glucose was abolished (at E) and remained abolished for about $1\frac{1}{2}$ hr. after the iodoacetic acid had been washed out, as seen at G and J. Between H and J glucose was given twice with the same result. The sole effect of glucose was inhibition of the spontaneous contractions. The inhibition was more pronounced in this experiment than in many others. A trace of inhibition was also seen at A, but was quickly overcome by the stimulating action of the glucose. It was obtained with pyruvate also, particularly when the latency for the stimulating action had lengthened. A similar initial inhibition has been described for other sugars [Feldberg & Solandt, 1942]. There was slight recovery at L which, however, did not develop in the following 2 hr., as seen by the effects of glucose at N and O.

dl-*Glyceraldehyde*

Like iodoacetic acid *dl*-glyceraldehyde inhibits the stimulating action of glucose and pyruvate, but, whereas iodoacetic acid inhibits glucose more completely and for a much longer period, the difference is less pronounced with glyceraldehyde. Complete recovery is obtained to the stimulating action of glucose when the glyceraldehyde is removed from the bath. In this respect it differs greatly from the more persistent effect of iodoacetic acid.

When added in a concentration of about 1 : 1000 to a preparation suspended in Tyrode solution containing glucose, glyceraldehyde causes gradual relaxation of the longitudinal muscle and reduction in the amplitude of its rhythmic contractions, but a few minutes after washing out the glyceraldehyde, even if it has been kept in contact with the muscle for more than an hour, recovery sets in and becomes complete within 30–40 min. On a preparation suspended

in glucose-free Tyrode solution glyceraldehyde has no depressing effect of its own, but inhibits the stimulating action of added glucose and pyruvate. In the experiment of Fig. 2, 0·5 mg. of sodium pyruvate (at A_1) and 20 mg. of glucose (at B_1) had about the same stimulating action. Glyceraldehyde was then added to the bath and left in contact with the muscle for 14 min. During this time glucose was given twice (B_2 and B_3) and pyruvate once (A_2). Full inhibition to glucose did not develop until the glyceraldehyde had been in contact with the
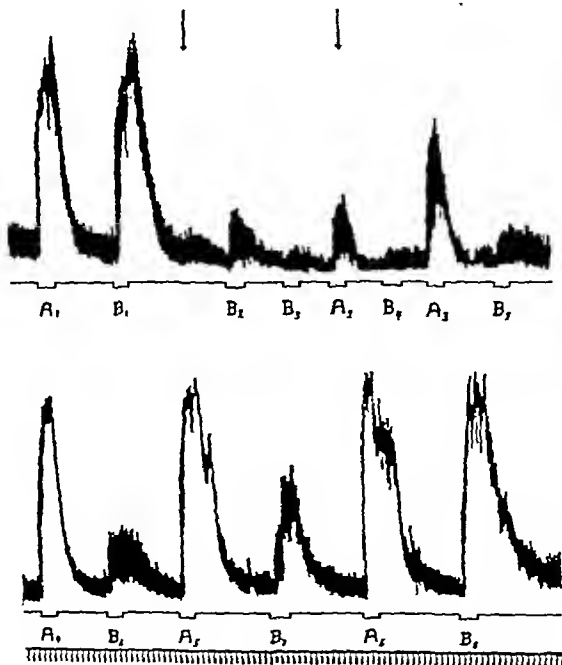
Fig. 2. Record of rabbit's small intestine preparation in 14 c.c. glucose-free Tyrode solution. At A_1–A_4 0·5 mg. sodium pyruvate; latencies 11, 18, 14, 14, 13 and 14 sec. respectively. At B_1–B_5 20 mg. glucose; latencies at B_1 21, at B_2 25, at B_6 25, at B_7 28 and at B_8 25 sec. Between the two arrows 10 mg. of dl-glyceraldehyde. Time in 30 sec. For details see text.

muscle for several minutes, but even then the effect of pyruvate was not abolished but greatly depressed only. After washing out the glyceraldehyde recovery to pyruvate proceeded quickly and was nearly complete in less than 20 min. (A_4). The contraction at A_4 differed from that at A_1 only by a longer latency and a more gradual development. Recovery to glucose took longer, but was complete within 40 min. after washing out the glyceraldehyde (B_8).

Weaker concentrations of glyceraldehyde than those used in the experiment of Fig. 2 had a comparatively weaker inhibitory action. For instance, 4 mg.

added to the 14 c.c. bath and left in contact with the muscle for several minutes, greatly depressed but no longer abolished the stimulating action of 20 mg. of glucose; this concentration already slightly inhibited pyruvate.

Phosphoglyceric acid, Cori ester, Embden ester and Harden-Young ester

None of these four phosphorylated compounds tested on the rabbit's intestine preparation, suspended in glucose-free Tyrode solution, could replace glucose or pyruvate, or at least to any appreciable extent. The results obtained with phosphoglyceric acid and Cori ester are illustrated by the experiment of Fig. 3.
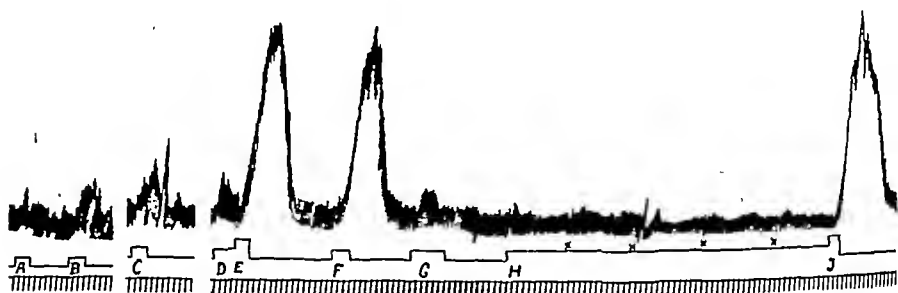
Fig. 3. Record of rabbit's small intestine preparation in 14 c.c. glucose-free Tyrode solution. At A 12 mg., at B 24 mg., at C 40 mg. and at D and G 20 mg. of the sodium salt of phosphoglyceric acid. At E, F and J 5 mg. of glucose. At H 100 mg. of the sodium salt of the Cori ester left in contact with the muscle for 30 min. At the crosses the fluid was washed out but replaced with glucose-free Tyrode solution containing 100 mg. Cori ester. Time in 30 sec. For details see text.

3-d-*Phosphoglyceric acid* had a slight stimulating action resembling that of glucose in so far as it started after a latency, but differing in so far as it subsided when the substance was left in contact with the muscle for a few minutes (at G). At A, B and C are seen the effects of 12, 24 and 40 mg. respectively of the sodium salt of phosphoglyceric acid left in contact with the muscle for 90 sec., and at G of 20 mg. left in contact for 3 min. The small stimulation of the longitudinal muscle started after a latency of 10–15 sec. There were strong constrictions of the circular muscle at C, observed directly through the glass wall of the vessel and due probably to the increase in tonicity [see Feldberg & Solandt, 1942]. Phosphoglyceric acid did not interfere with the action of glucose. At D, 20 mg. of the phosphoglyceric acid, and 2 min. later without washing it out, 5 mg. of glucose were given at E, both substances being washed out 90 sec. later. The stimulating effect of glucose resembled that at F, when given alone; in both there was a latency of 25 sec.

Cori ester (glucose-1-phosphate), given at the end of the tracing, had no effect at all. From H onwards the bath contained, for 30 min., 100 mg. of the ester. The ester did not influence the response to glucose, 5 mg. of which were given at J, without washing out the ester. After a further period of 90 sec. both

substances were washed out. The latency was 25 sec. and the effect somewhat stronger and longer lasting than at *E* and *F*. Such an increase in the stimulating action of glucose is often observed with its repeated administration, particularly if there had been a long interval since the last dose was given.

Embden ester (*hexose-6-phosphate*) was tested in one and *Harden-Young ester* (*fructose-1, 6-diphosphate*) in two experiments. Embden ester produced slight immediate and short-lasting contraction of the longitudinal muscle, and, in stronger concentrations, contractions of the circular muscle. The effects were probably not specific, but due to impurities with barium or sulphate ions. At least the effects did not resemble those of glucose. The Harden-Young ester appeared to produce a slight glucose-like effect if added to the bath in a dose equivalent to 10 mg. of hexose or more. The effect was not obtained regularly, but might indicate that this ester, to a slight extent, was able to replace glucose.

DISCUSSION

Our results show that iodoacetic acid may poison smooth muscle in such a way as to render it insensitive to the stimulating action of glucose, but not to that of pyruvate. This condition is obtained when the iodoacetic acid is washed out after a few minutes' contact with the muscle. Nevertheless, iodoacetic acid depresses the effect of pyruvate, although to a lesser degree than that of glucose. Contrary to our results Prasad [1935] observed incomplete recovery only to pyruvate of the intestinal muscle poisoned with iodoacetic acid, probably because he left the acid in contact with the muscle throughout the experiment. In explaining the mechanism of iodoacetic-acid poisoning on the carbohydrate metabolism of smooth muscle the fact has to be taken into account that it influences the stimulating action of glucose as well as that of pyruvate although in different degrees. Since lactate, added to smooth-muscle extracts, is not oxidized [Prasad, 1935] and cannot replace glucose or pyruvate in the smooth-muscle preparation [Feldberg & Solandt, 1942], the analysis of the inhibitory action of iodoacetic acid could not be extended to this derivative of carbohydrate metabolism. The isolated frog's heart, poisoned by iodoacetic acid, on the other hand, is able to use not only sodium pyruvate but also sodium lactate, methyl glyoxal [Clark, Eggleton, Eggleton, Gaddie & Stewart, 1938] and phosphoglyceric acid [Parnas, Ostern & Mann, 1934].

The inhibition produced by glyceraldehyde on the stimulating action of glucose on the smooth-muscle preparation accords with its known inhibition on glycolysis in minced tissue and tissue slices. In our experiments it also inhibited, although to a lesser degree, the stimulating action of pyruvate which revives the inhibition of glycolysis in tissue slices or tissue extracts. The gradual but complete reversibility of the inhibition produced by glyceraldehyde on the smooth-muscle preparation, when washed out of the bath, may be associated with a slow conversion into lactic acid of the glyceraldehyde not washed out

from the muscle. Proof for such a conversion has been given by Needham & Lehmann [1937].

The inability to replace, in the surviving smooth muscle, glucose or pyruvate by phosphorylated hexose esters, or to replace them to any appreciable degree, may result from the fact that the living cells are relatively impermeable to them. A similar interpretation has been given by Kosterlitz [1942] for the slow rate of fermentation of phosphorylated compounds by living yeast cells. Since phosphoglyceric acid appears to be utilized by the frog's heart, even when poisoned with iodoacetic acid, its inability to replace glucose to any extent in the intestinal muscle could be explained in a similar way only by attributing to the intestinal muscle a permeability to carbohydrate derivatives different from that of the heart muscle. There is no evidence for such an assumption.

SUMMARY

On the isolated rabbit's intestine preparation suspended in glucose-free Tyrode solution the following results were obtained:

(1) Iodoacetic acid and *dl*-glyceraldehyde inhibit, on the longitudinal muscle, the stimulating action of glucose and to a lesser extent that of pyruvate. After washing out the iodoacetic acid the muscle remains more or less insensitive to glucose but becomes, after a short time, fully sensitive to the stimulating action of pyruvate. After washing out the glyceraldehyde complete recovery takes place to pyruvate as well as to glucose, recovery to pyruvate, however, occurring earlier than that to glucose.

(2) The phosphorylated compounds, phosphoglyceric acid, Cori ester, Embden ester and Harden-Young ester, cause only a slight glucose-like stimulation of the longitudinal muscle or none at all.

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PANCREOZYMIN, A STIMULANT OF THE SECRETION OF PANCREATIC ENZYMES IN EXTRACTS OF THE SMALL INTESTINE

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The external secretion of the pancreas has, since the work of Mellanby [1925], been regarded as under a dual hormonal and nervous control: secretin controlling the amount and bicarbonate content of the juice, the vagus nerve its enzyme content.

Several observers, however, have reported that secretin itself stimulates enzyme production. Chiray, Jeandel & Salmon [1930] found that intravenous injection of secretin in man not only increased the rate of flow of pancreatic juice, but also increased the concentration of enzymes in the juice two- or three-fold. Hammarsten, Ågren & Lagerlöf [1937] also seemed at one time to regard secretin as an enzyme stimulant, but in a more recent paper Lagerlöf [1939] concludes that 'it is unlikely that the secretin produces any increased activity within the enzyme-producing cells'. Voegtlin, Greengard & Ivy [1934] observed no consistent increase in the concentration of enzymes in the pancreatic juice following intravenous injections of secretin in dogs and man. Barrington [1941], on the other hand, using secretin prepared by Ivy's method, concluded that secretin directly stimulated the discharge of pancreatic enzymes. Harper & Vass [1941] found that in cats the passage of foodstuffs or water through the pylorus into the duodenum, or their injection into the duodenum, resulted in an increase in the enzyme output by the pancreas, even when all the extrinsic nerves to the small intestine had been cut—an observation which cannot be reconciled with Mellanby's theory of a vagal control of enzyme output. Harper & Vass also found that secretin, prepared by Mellanby's method [Mellanby, 1932], had no stimulant action upon the output of enzymes from the pancreas.

It seems clear therefore that subsequent work has not supported Mellanby's sharp demarcation of hormonal and nervous control of pancreatic secretion. On the one hand several observers have claimed that secretin stimulates the production of pancreatic enzymes; on the other hand Harper & Vass observed increases in the enzyme output when the vagus nerves had been cut.

Many of the discrepancies between these various observations could be explained if there were present in the intestinal epithelium a hormone capable of stimulating the production of enzymes by the pancreas. It was to the examination of this possibility that the following experiments were directed.

METHODS

The experiments were performed on cats, anaesthetized with chloralose (0·08 g./kg. body weight, intravenously). The animals were fed a few hours before the experiment. The splanchnic nerves were cut extraperitoneally and pancreatic juice was collected by a cannula inserted into the pancreatic duct. To preclude stimulation of the pancreas by bile or acid in the duodenum, the bile duct and the pylorus were occluded.

A continuous flow of pancreatic juice of low enzyme content was maintained by regularly repeated injections of secretin prepared by Mellanby's method. The amount of secretin injected was sufficient to maintain a rate of flow of about 1·0–1·5 c.c. in 10 min. The amylase content of successive samples of juice was measured by Wohlgemuth's method, and in a few experiments the trypsinogen content was measured by the formaldehyde titration method. The methods used were those given in detail by Harper & Vass [1941].

From the amylase and trypsinogen contents of the various samples the minute output of these enzymes can be calculated, by multiplying the content by the amount of the sample in c.c. divided by the time of collection of the sample in minutes. In the figures illustrating this paper the term D_T^J refers to the minute output of amylase calculated in the above manner. The rate of secretion is expressed as the number of c.c. of juice secreted in 10 min.

RESULTS

Preparations have been made from extracts of the mucosa of the small intestine and have been tested by intravenous injection into cats. Against the background of a steady flow of pancreatic juice of low enzyme content it has been possible to observe the effect of these upon the rate of flow of juice and the output of enzymes by the pancreas. By this method we have obtained preparations which increase the output of enzymes from the cat's pancreas without having any secretin activity (Fig. 1). For the active substance producing this effect we suggest the name 'Panoreozymin'.

Method of preparation of pancreozymin

The principle of the method is as follows: the secretin and panoreozymin are extracted from the intestinal mucosa with alcohol. After distilling off the alcohol the secretin is removed by Mellanby's bile-salt method, and the pancreozymin is then precipitated by the addition of NaCl.

The mucous membrane is scraped from the first 3–4 ft. of the small intestine of the pig, ground with sand and extracted for $\frac{1}{2}$ hr. with four times its weight, expressed in c.c., of 99% alcohol, the mixture being shaken gently during the extraction. The extract is then filtered. If not used immediately the filtrate is kept in the ice-chest in dark bottles. It is next mixed with an equal volume of
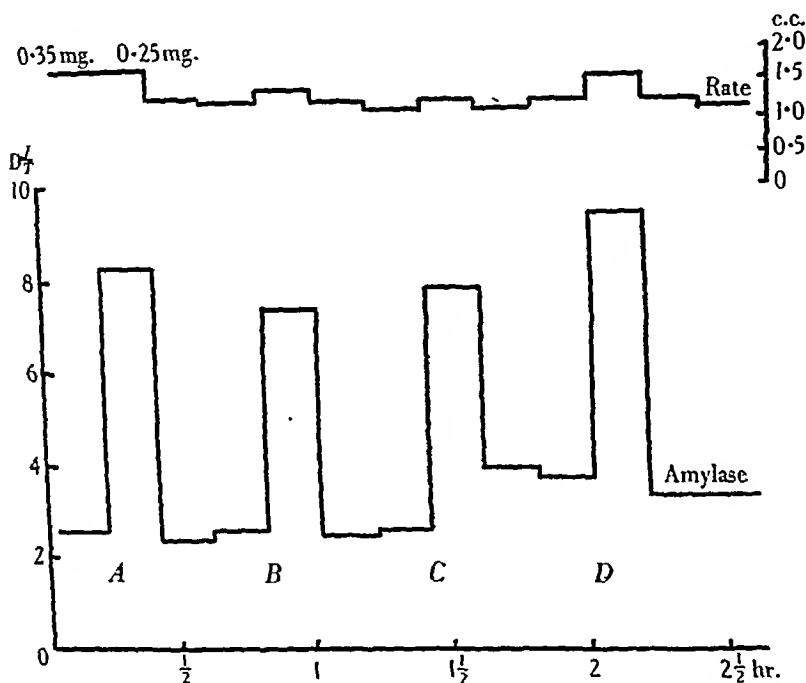
Fig. 1. The flow of pancreatic juice was maintained by the injection at intervals of 12 min. of 0·35 mg. secretin for the first two injections, and 0·25 mg. for all the later samples. In addition 7·5 mg. of a pancreozymin preparation ('NaCl precipitate') were injected at A, B, C and D. Following each of these injections there was a well-marked increase in the minute output of amylase. The pancreozymin injected at A and at B had been dissolved 20 hr. previously in 0·1 N HCl and 0·1 N NaOH respectively and kept in the ice-chest. The sample injected at C had been heated in a boiling water bath for 30 min. before injection. None of these procedures affected the activity of the pancreozymin.

aqueous 0·1 M CaCl$_2$ to precipitate lipoidal material, allowed to stand for $\frac{1}{4}$–$\frac{1}{2}$ hr. and filtered. Five hundred c.c. portions of the filtrate are concentrated under reduced pressure (outer bath temperature not exceeding 40° C.) to one-quarter its bulk. The secretin is precipitated by adding to each 500 c.c. of the concentrated solution 1 g. bile salt (commercial tauroglycocholate) dissolved in about 10 c.c. water, followed by 15 c.c. 1% acetic acid. The secretin is separated in a centrifuge and may be purified by extracting it with a small volume of absolute alcohol and precipitating with acetone. A trace of CaCl$_2$ assists

flocculation of the secretin at this stage. The supernatant fluid obtained after centrifuging off the secretin is saturated with sodium chloride and allowed to stand in a round flask in the dark for 2–3 days. By this time a sticky precipitate has settled out on the bottom and sides of the flask. It consists partly of bile salt and contains the pancreozymin. The liquid is poured off and rejected. The precipitate is dissolved in a small volume of water, filtered and evaporated to dryness in a vacuum desiccator over H_2SO_4. We have called this preparation the 'NaCl precipitate'. It contains a considerable amount of NaCl. One g. of the residue is shaken with 50 c.c. absolute alcohol for an hour in a shaking machine. This dissolves the active substance. The solution is filtered and taken almost to dryness by distillation under reduced pressure. The residue is dissolved in a little water and evaporated to dryness in a vacuum desiccator. This is the material with which most of our experiments have been done. It contains little or no secretin, but causes a marked increase in the enzyme concentration of pancreatic juice when injected intravenously into cats. We have called it the 'alcohol-soluble' preparation. It may be purified further in the following way. Fifty mg. are mixed with 0·2 c.c. N HCl and 2 c.c. pure acetone. When all has dissolved 30 c.c. pure, dry acetone are added, and after standing an hour the precipitate is centrifuged off. It is washed with dry acetone and then dry ether, and dried in a vacuum desiccator. Some of the activity is lost in this process, but the product is about twice as active weight for weight as the 'alcohol-soluble' preparation. It is very hygroscopic. We have called it 'preparation A.A.'

Attempts have been made to purify pancreozymin preparations by adsorption. The pancreozymin preparation was dissolved in water, shaken with the adsorbent for 15 min., centrifuged and the supernatant fluid tested. No diminution in activity was observed with the following adsorbents: kaolin, franconite, aluminium hydroxide $C\gamma$ or benzoic acid. A certain amount of success was obtained with colloidal ferric hydroxide. Using a solution of the initial 'NaCl precipitate', all the activity was removed by precipitation with B.P. 'dialysed iron' and recovered completely by elution with 0·25% aqueous ammonia. This resulted in a trebling of the activity per mg. dry substance. On a larger scale, however, the process was not so effective and it obviously requires further investigation. The most active material per mg. dry weight has been obtained by precipitating a 1% solution of the 'alcohol-soluble' preparation containing a trace of NaCl with 10% trichloroacetic acid, centrifuging, and washing the precipitate with acetone and ether. By this method, however, about half the total activity is lost. Attempts to obtain more active preparations by the use of Reinecke salt or phosphotungstic acid have so far not been successful.

Activity of pancreozymin preparations

We have not attempted to express the activity of pancreozymin preparations in terms of 'units', as we are not convinced of their value either in the case of pancreozymin or of secretin. As a rule we have not accepted the response to a pancreozymin preparation as positive unless the sample collected during the 12 min. following the injection showed a minute output of amylase twice that of the control sample collected for 12 min. before the injection. Average figures from 50 experiments show that after the injection of 'NaCl precipitates' in doses of 2 mg./kg. body weight the minute output of amylase rose to 12·2, compared with a control level of 4·8. The injection of 'alcohol-soluble' preparations in doses of 1 mg./kg. body weight increased the minute output of amylase to 13·9 from a control level of 6·3.

On an average 100 g. intestinal mucosa gives a yield of 300–400 mg. 'NaCl precipitate' or 100–150 mg. 'alcohol-soluble' preparation. An effective dose of pancreozymin contains the active material from 1·5 to 2·5 g. intestinal mucosa.

Mode of action of pancreozymin

That the response to pancreozymin is not secondary to any action, either central or peripheral, upon the vagus nerves is shown by the fact that pancreozymin is active in vagotomized animals and after the intravenous injection of atropine (Fig. 2).

Most of the experiments were done on animals fed a few hours previously, but the pancreatic action of pancreozymin cannot be secondary to any effect upon the intestinal mucosa, facilitating the absorption of intestinal contents, as injections of pancreozymin are effective in starved as well as in fed animals.

In a few experiments the trypsinogen content of the pancreatic juice was estimated in addition to the amylase, and it was found that pancreozymin, like stimulation of the vagus nerve, produces a parallel increase in the output of amylase and trypsinogen. Repeated injections of pancreozymin over a period of 1 hr. show no diminution in the response of the pancreas in the later samples.

The action of pancreozymin upon the pancreas cannot be accounted for by any vasodilator material in the extracts. Cruder preparations of pancreozymin ('NaCl precipitate') have a slight evanescent depressor effect upon the arterial blood pressure, but purer preparations are free from such activity. A sample of pancreozymin was assayed for histamine by the guinea-pig ileum method. The assay showed 'no evidence of histamine', i.e. an effective dose of pancreozymin contained less than 7 μg. of histamine. The intravenous injection of this amount of histamine produced a more definite fall of arterial blood pressure than a crude pancreozymin extract, but had no effect upon the enzyme content or

rate of flow of pancreatic juice. Intravenous injections of pancreozymin are without effect upon the level of the blood sugar either in chloralosed cats or in unanaesthetized rabbits.
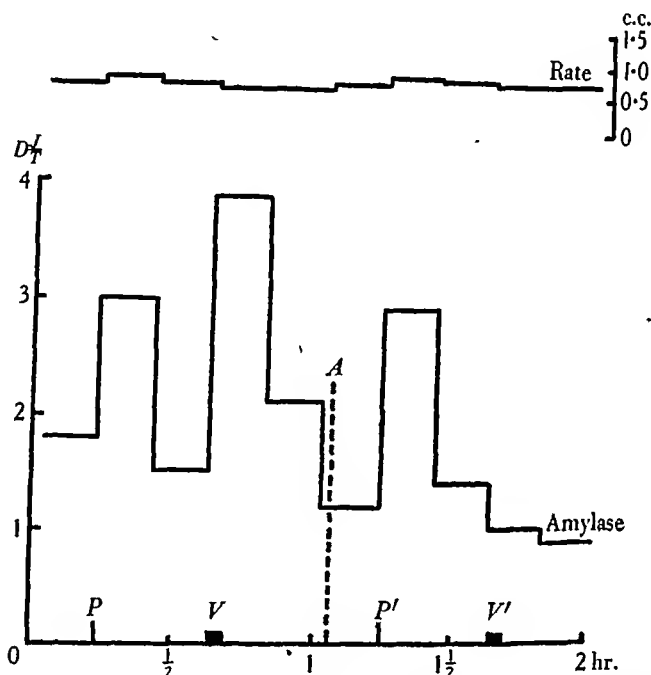
Fig. 2. The splanchnic nerves were cut extraperitoneally, and the dorsal vagus trunk was cut on the oesophagus. An injection of pancreozymin (5·5 mg. 'alcohol-soluble' preparation) at P, and faradic stimulation of the dorsal vagus trunk for 4 min. at V, were each followed by an increase in the minute output of amylase. After the intravenous injection of 1·25 mg. atropine sulphate at A, a second injection of pancreozymin at P' produced an increase in the minute output of amylase, but a repetition of the vagal stimulation at V' was without effect.

Chemical characteristics of pancreozymin

The 'NaCl precipitate' or 'alcohol-soluble' preparations retain their activity for many months when kept in the dry state. The activity of an 'alcohol-soluble' preparation was not affected by standing at room temperature overnight in N_1 HCl, but it disappeared completely under the same conditions with N_1 NaOH. On the other hand, 15 mg. of the 'NaCl precipitate' dissolved in 2 c.c. $N/10$ NaOH and kept overnight in the ice-chest maintained its activity fully (Fig. 1).

To test the action of pepsin on pancreozymin three tubes were set up as below:

	1	2	3
'Preparation A.A.'	5·1 mg.	5·1 mg.	—
0·2 % pepsin (Merck)	0·5 c.c.	—	0·5 c.c.
N_1 HCl	0·2 c.c.	0·2 c.c.	0·2 c.c.
H_2O	1·5 c.c.	2·0 c.c.	1·5 c.c.

The tubes were incubated at 37° C. for $17\frac{1}{2}$ hr. It was confirmed that in this time the concentration of pepsin used was sufficient to dissolve fibrin. After incubation the liquid in each tube was neutralized with NaOH, brought to 3 c.c., and 2 c.c. used for testing by intravenous injection. This showed that the same amount of pancreozymin was present in 1 and 2 and that the pepsin itself (tube 3) had no pancreozymin activity. Pancreozymin is therefore not digested by pepsin. To test the stability of pancreozymin to pancreatic juice, 0·1 c.c. cat's pancreatic juice activated by enterokinase was added to 2 mg. 'preparation A.A.' in 2 c.c. phosphate buffer pH 7·4. The pancreozymin activity was completely lost in $2\frac{1}{2}$ hr. at 37° C. Crude pancreozymin appears to be thermostable, for a solution of 15 mg. in 4 c.c. water lost no activity on heating in a boiling water-bath for $\frac{1}{2}$ hr. (Fig. 1).

To test dialysability a solution of the NaCl precipitate in water was allowed to dialyse into water in the ice-chest using a collodion sac. After 24 hr. both inner and outer solutions were found to be inactive. A small amount of active material was obtained by extracting the collodion with alcohol, indicating that adsorption was the probable cause of lack of activity. Using a cellophane membrane under similar conditions it was found that dialysis occurred but was far from complete in 24 hr., although three changes of the outer water had been made. This suggests that the molecule of pancreozymin is about the same size as a proteose.

Pancreozymin in secretin preparations

It was maintained by Mellanby [1925] that preparations of secretin made by his bile-salt method contain no substance which stimulates enzyme secretion. On the other hand, Hammarsten *et al.* [1937] and Barrington [1941] have suggested that secretin does stimulate enzyme production. Barrington used a preparation made by the method of Greengard & Ivy [1938] which they designate S.I. This secretin is made by extracting the intestinal mucosa with 0·4 % HCl, saturating with NaCl and extracting the resulting precipitate with 90 % alcohol. The alcohol is removed by distillation, and after adjusting the pH to about 5·4 the liquid is warmed to precipitate inactive protein. From the filtrate the secretin is precipitated by the addition of trichloroacetic acid. We have confirmed that S.I. preparations made in this way contain both secretin and pancreozymin, since on intravenous injection they cause an increased output of both pancreatic juice and enzymes.

We have, however, been able to show that if the extraction is made as described by Greengard & Ivy, but the final precipitation is made with bile salt and acetic acid instead of trichloroacetic acid, then a very active secretin may be obtained which is free from pancreozymin. To obtain this secretin the bile-salt precipitate is extracted with a small volume of alcohol, leaving some undissolved material, and the extracted secretin precipitated with acetone. We have called this secretin S.II. (Fig. 3). The substance in the precipitate ob-

tained by bile salt and acetic acid which does not dissolve in alcohol was found to contain pancreozymin. After the crude secretin has been removed by bile salt and acetic acid, the supernatant fluid if saturated with NaCl yields more pancreozymin. This remains undissolved with some NaCl when the NaCl precipitate is extracted with absolute alcohol.

Fig. 3. Separation of pancreozymin and secretin. The flow of pancreatic juice was maintained by the injection at intervals of 12 min. of 0·4 mg. of Mellanby's secretin, except at *A* and *B*. At *A*, instead of Mellanby's secretin, 1·6 mg. of an S.I. preparation (containing secretin and pancreozymin) were injected. This was followed by a marked increase in the rate of flow of juice, and an increase in the concentration of amylase (i.e. an increase in the diastatic index). The minute output of amylase was consequently also greatly increased. At *B* 0·28 mg. of S.II. (containing secretin only) was injected. The increase in the rate of flow of juice which followed was the same as at *A*, but there was no increase in the concentration of amylase. There was therefore only a negligible increase in the minute output of amylase.

The pancreozymin preparation obtained as above from the original extract made with 0·4 % HCl thus differs from that made by direct alcohol extraction of the intestinal mucosa in being insoluble in alcohol. It seems to us possible that this difference in properties may be caused by heating the acid liquid containing the secretin and pancreozymin at pH 5·0–5·4 to cause flocculation of inactive protein, this being a step in the preparation of secretin S.I. by the

Greengard & Ivy method. We intend to investigate this problem as soon as opportunity permits.

We have tested the secretin preparation of Hammarsten and his colleagues, called 'Pancreotest', and find that like Mellanby's secretin it contains no pancreozymin.

Distribution of pancreozymin

Most of our preparations have been made from the uppermost 3–4 ft. of pig's small intestine, but equally active ones have also been obtained from cat and dog intestines. In one experiment using the relatively short intestine of the dog the activity of preparations made from the upper half and the lower half was about equal. With the cat the upper half gave a slightly more active preparation than the lower half. In an experiment in which six pig intestines were used, extracts were made from the uppermost 4 ft. and the lowermost 4 ft. The latter was quite inactive, though the former was very active. Preparations made from the stomach of pig, cat and dog were found to possess no activity. It may thus be concluded that the distribution of pancreozymin in the small intestine is similar to that of secretin. Incidentally, the negative experiments with gastric mucosa serve as a control, since the method of extraction and precipitation with bile salt was identical with that which gave active preparations from the small intestine.

DISCUSSION

The available evidence suggests that pancreozymin acts directly upon the cells of the pancreas. The activity of pancreozymin in atropinized animals eliminates the possibility that the vagus is involved, and its effectiveness in starved animals makes it unlikely that the stimulation of the pancreas is secondary to a dilator action on the vessels of the intestinal mucosa, allowing of the absorption of secretagogue substances. Much of the earlier opposition to the acceptance of the physiological importance of secretin was based on the argument that the vasodilator action of intestinal extracts was responsible for the effect on the pancreas [Still, 1931]. Pancreozymin extracts, however, have little or no depressor effect on the arterial blood pressure, and small amounts of histamine have no pancreozymin activity. The S.I. preparation of Greengard & Ivy is also practically free from vasodilator material.

The stimulant action of pancreozymin upon the pancreas appears to be confined to the enzymes of the external secretion. Occasionally preparations of pancreozymin show very slight secretin activity, but the majority have no effect on the rate of flow of the juice. Various workers have claimed to extract from the intestinal mucosa material which stimulates the production of insulin by the pancreas, and the evidence on the subject has recently been summarized by Loew, Gray & Ivy [1940]. Pancreozymin preparations have no such hypoglycaemic action.

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The attribution of the property of stimulating enzyme production to secretin by some authors is caused by the contamination of their secretin preparations with pancreozymin. We have shown that the secretin preparation of Greengard & Ivy which they call S.I. contains pancreozymin, and by modifying their method of preparation in the final stages it is possible to obtain secretin free from pancreozymin. The argument might be put forward that the secretin molecule had been broken up by the method of extraction used by us and the part stimulating enzyme production (pancreozymin) had been split off. But a simple extraction with alcohol followed by precipitation with bile salt in a faintly acid solution is not likely to cause such a change.

It is clear that further investigations must be carried out to determine the best conditions for extracting pancreozymin and purifying it. All we have been concerned to show at present is that this substance exists side by side with secretin and will therefore be extracted along with the latter in making the crude extracts from which secretin is later prepared.

Is pancreozymin a hormone of physiological importance, or is it merely of pharmacological interest? In favour of a physiological status are its freedom from any generalized vasodilator activity and the close parallelism between its distribution in the gastro-intestinal tract and that of secretin. The acceptance of pancreozymin also provides the simplest explanation of the observation of Harper & Vass that the injection of water into the duodenum increases the pancreatic enzyme output of animals which have had the extrinsic nerves to the intestine cut. It would explain too the observation of Farrell & Ivy [1926] that following the administration of meals to dogs the juice from a transplanted pancreas increased not only in quantity but in amylase content.

If pancreozymin is accepted as of physiological importance, Mellanby's conception of an exclusively nervous control of enzyme secretion must be abandoned. By this we do not imply either that vagal control of enzyme secretion does not exist or that it is unimportant. Many observers have demonstrated that there are fibres in the vagus nerves which on electrical stimulation increase the production of enzymes by the pancreas. There is still little or no information as to how a discharge of impulses in these fibres is brought about in the normal animal.

SUMMARY

1. From the small intestine of the pig, dog, and cat a substance has been extracted which on intravenous injection into cats causes an increased secretion of enzymes by the pancreas but has no effect on the volume of juice secreted. We have named this substance 'pancreozymin'.

2. A method is described whereby pancreozymin can be separated from secretin, which accompanies it in alcoholic extracts of the small intestine.

. Pancreozymin is thermostable. It is stable in acid but not in alkaline ition. Pepsin does not affect it but it is destroyed by pancreatic juice. It lyses slowly through cellophane.

. The response of the pancreas to pancreozymin is unaffected by section of vagus and splanchnic nerves or by the administration of atropine.

5. The distribution of pancreozymin in the small intestine is similar to that secretin. It is not present in extracts of gastric mucosa.

6. It is suggested that there may be a dual nervous and hormonal control of e secretion of enzymes by the pancreas and that pancreozymin is the hormone sponsible for the latter.

7. Preparations of pancreozymin do not owe their action to vasodilator ibstances and have no hypoglycaemic action.

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CAPILLARY PERMEABILITY IN TRAUMATIC SHOCK

By D. ENGEL and E. FORRAI, *From the Department of Neuro-surgery of the Royal Infirmary, Edinburgh, and the Department of Physiology, Royal (Dick) Veterinary College, Edinburgh*

One of us [Engel, 1940] has recently worked out a method for determining directly the permeability of the capillaries in a given region. This method appeared suitable for reinvestigation of the problem of increased permeability of capillaries in 'traumatic shock'. We have therefore examined whether, after crushing a limb, there occurs an increase in the filtration through the capillaries either confined to the traumatized region and its immediate neighbourhood or generalized throughout the body.

In traumatic shock a great quantity of plasma is assumed to escape from the blood vessels owing to their increased permeability. Evidence for this loss of plasma is of a twofold nature: the haemoconcentration found in man as well as in experimental animals during traumatic shock and the increase in weight of a traumatized limb. Both findings, however, are open to a different interpretation. Haemoconcentration may be due to escape of fluid constituents of blood, but it may also occur as a result of mobilization of blood corpuscles from the blood reservoirs, especially the spleen and the bone marrow. This possibility has not always been excluded as a cause of the haemoconcentration. The increase in weight of a traumatized limb, found by Blalock [1930] has been contested by some [Moon, 1938] but confirmed by others [Phemister, 1928; Harkins, 1941]. It results from loss into the crushed limb out of the vessels, of plasma as well as blood, in amounts sufficient to account for death. It is not possible to determine to what extent the increase in weight is due to escape of whole blood from broken or torn vessels or to escape of fluid through the capillary walls. Beard & Blalock [1931], by centrifuging crushed tissue in order to separate the extravascular fluid, attempted to show that the oedema fluid of a crushed limb has a similar composition as serum. This method, however, does not differentiate between the plasma of the extra- and intravascular blood in the tissue and the extravascular oedema fluid. Both will appear in the supernatant fluid on centrifugation. There is thus no direct evidence of an increased capillary permeability in shock.

Neither is there any clear evidence if the accepted increased permeability of the capillaries is generalized or confined to the traumatized area. Blalock [1940], in criticizing the toxaemic theory, concludes that 'there is no convincing proof that local injury in itself produces general damage to all capillaries and tissues'.

METHODS

The experiments were carried out on cats, with the exception of one experiment which was performed on a rabbit. The animals were anaesthetized by intramuscular injection of 0·5 c.c./kg. of nembutal (for veterinary purposes) about 1 hr. before the actual preparation was started. A cannula was tied into the carotid artery for recording the arterial blood pressure. Chlorazol fast pink (0·1 g./kg.) was injected intravenously as an anticoagulant. At different times about hourly, the haemoglobin concentration of blood samples taken from the otherwise unused jugular vein was determined with the Sicca haemoglobinometer.

Determination of rate of filtration through the capillaries. A dye was injected into the jugular vein at a constant rate throughout the experiment, while the two knee joints were perfused with Ringer solution. The dye circulating in the blood will appear in the perfusate of the knee joints after it has passed the capillary endothelium, the synovial membrane and the fine layer of tissue connecting the two. We measured the filtration through this composite membrane, which will be referred to as the articular barrier. A diagrammatic representation of the barrier and its relation to the perfusion is given in Fig. 1B. The apparatus for the perfusion of the knee joints and the infusion of the dye is illustrated in Fig. 1A. It consisted of two identical record syringes of 10 c.c. and a third syringe of 20 c.c., which were mounted firmly side by side, and the pistons of which were pushed forward simultaneously by a metal wheel (*w*) so as to empty the three syringes at an identical steady rate. The propelling of the wheel was effected by a motor as described elsewhere [Engel, 1941]. The 20 c.c. syringe, connected by rubber tubing (*a*) to a cannula tied into the jugular vein, contained a dye solution, usually of 1 % acid fuchsin, which was infused at a rate of about 0·7 c.c./min. Each 10 c.c. syringe was connected by rubber tubing (*b*, *b₁*) with a needle inserted into the left and right knee joint respectively, medial to the patella. These syringes were filled with Ringer solution which was warmed in its passage along the rubber tube by an electrically heated jacket (not indicated in the figure). A needle was inserted into each knee-joint cavity lateral to the patella allowing the fluid to flow out of the cavity (small arrows in Fig. 1), which in a cat held between 2 and 4 c.c. of perfusate. Care was taken to avoid bleeding in order to keep the articular barrier intact. Only perfusates free from blood were considered satisfactory and, at the end of each experiment, it was ascertained whether the tips of the needles had been in the joint cavities. The perfusates were collected separately

from each knee joint every 20–30 min. and the dye content was determined colorimetrically. The syringes were emptied in about half an hour.

The dye concentration in the blood was estimated every hour. 0·5 c.c. blood was removed from the jugular vein not used for the dye infusion, diluted to 1/200 and centrifuged. The supernatant fluid was compared colorimetrically with a standard dye solution after addition of a few drops of acetic acid to regenerate the acid fuchsin (a procedure followed also with the perfusates). The solution was the same as used for the perfusates from the knee joints (1/400,000). (The dye concentration was expressed in millimetres. as read on the colorimeter.) In any one experiment the same quantity of the perfusate
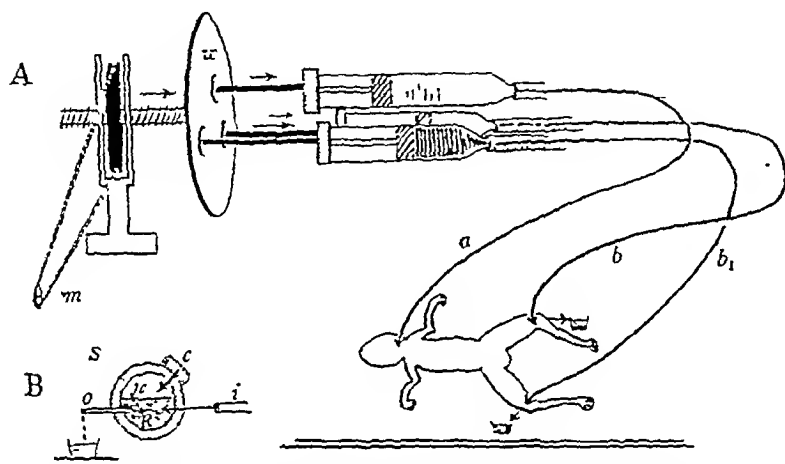
Fig. 1. A. Arrangement for knee perfusion. w, wheel pushing the three pistons; a, tubing connected with jugular vein; $b-b_1$, tubing connected with the two knee joints. The two small arrows represent the cannulae emptying the perfused joints. (For details see text.) B. Showing the dialysing process from one capillary (c), supplying the synovial membrane (S), into the joint cavity (jc) which is perfused with Ringer solution (R). i, inflow; o, outflow; m, motor.

—10–40 mm. high column as read on the colorimeter—was compared with the standard solution, recorded again in millimetres, and charted so in the curves. The figures obtained are relative values, but the absolute quantities can easily be calculated from them.

In addition to measuring the dye excretion into the knee joints we have measured the temperature of the quadriceps muscles with two copper-constantan thermo-couples, each inserted into a serum needle, by the usual thermo-electric method [for details see Engel, 1941].

Crushing the limbs. In one series of experiments one of the hind legs, including the foot, was traumatized before or during the perfusion of the knee joints, the other leg serving as a control. At the end of the experiment the two hind legs were weighed after dividing the pelvis in the midline according to the technique of Blalock [1930]. When the crushing was performed several hours

before the perfusion of the knees it was done under a short ether anaesthesia The trauma consisted of 80–100 hammer blows to the soft parts of the extremi ties, usually to the parts distal to the knee joints, but in a few experiments the region of the thigh was traumatized either alone or in addition. In another series of experiments both forelegs and feet were similarly traumatized during the perfusion of the knee joints, and the dye excretion of both was compared before and after crushing. In most experiments the tibia, fibula and the tarsa bones of the hammered limbs were also fractured. Care was taken not to break the skin, but some slight bleeding could not always be avoided.

RESULTS

In animals without trauma the dye excretion from the perfused knee joint was practically identical on both sides and increased gradually in the course o: the experiment, with the gradual accumulation of dye in the blood. The dye excretion was, approximately proportional to the blood dye concentration, variations being due to various factors which were out of control (pH of body tissue, renal and liver excretion of dye, etc.). The temperature of the two quadriceps muscles did not differ by more than $0\cdot1$–$1\cdot0°$ C. It usually decreased considerably due to the slow general cooling caused by the nembutal.

Crushing one hind leg

This was followed by considerable swelling of the limb which at the end of the experiment, lasting about 6 hr., weighed 50–130 g. more (average 90 g. or 3 % of the body weight) than the uncrushed limb of the other side. The crushing caused first a very marked increase and later a decrease of dye excretion from the perfused knee joint of the crushed leg in comparison to the excretion from the control side. The increased excretion started a few minutes after the crushing and was detectable in the sample of perfusate collected during the first 20 min. after the trauma. In twelve experiments (eleven cats, one rabbit) the increased dye excretion, when at its maximum, was 2–7 times (average 3·5 times) greater than the excretion from the control leg. This increase lasted from 1 to 3 hr. and was followed by decreased excretion, continuing usually to the end of the experiment. Only when the animal died during the perfusion did the excretion rise again to some degree shortly before death, probably on account of a general anoxia.

In those experiments in which no blood pressure was recorded the dye excretion before the trauma was observed for a longer period. The excretion increased gradually on both sides with the increased dye concentration in the blood. The trauma was often followed by a drop in the excretion of the non-traumatized side. This drop amounted sometimes to over 25 %, while there was a concurrent increase of dye excretion of 100 % on the traumatized side.

A typical experiment illustrating the initial increase and later decrease of dye excretion after trauma is given in Fig. 2 C. The immediate increase was followed
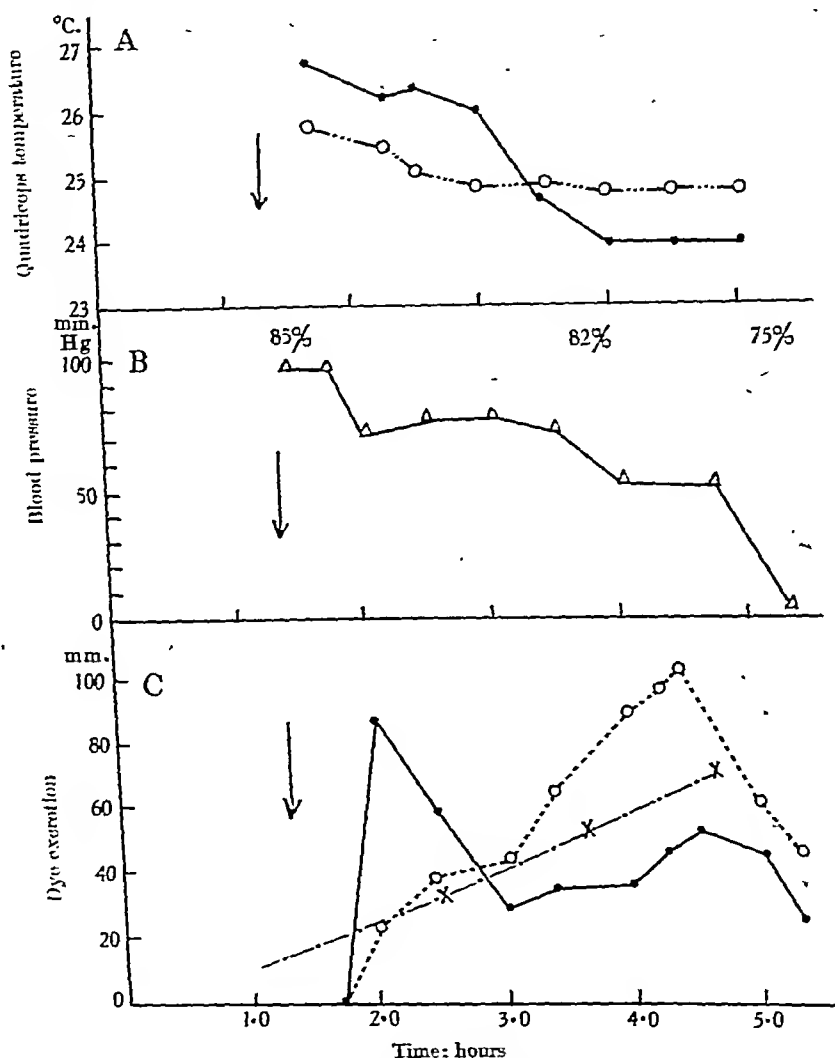
Fig. 2. Effect of crushing right hind leg on muscle temperature (A), blood pressure (B), and dye excretion (C). In A and C continuous line, right crushed; dotted line, left hind leg. Interrupted line in C, dye concentration of blood (reduced to 1/200). The arrows indicate time of crushing. Abscissae: time in hours. The figures on top of B are haemoglobin concentration of venous blood.

after 2 hr. by a decrease below the level of the control side despite the rise in blood-dye concentration. The blood pressure in this experiment is given at B, the figures on top of the tracing are haemoglobin concentrations of the blood

at the times indicated. In this and many other experiments, the blood pressure, although falling gradually, was maintained on a relatively high level for a considerable time before the final collapse occurred with heart failure, preceded by a period of Cheyne Stokes's respiration. No absolute haemoconcentration has been observed in this or any of the other experiments, the haemoglobin percentage actually fell below the pretraumatic level. This absence of haemoconcentration may have resulted from the severity of the trauma and the consequent considerable loss of whole blood from the torn vessels as witnessed by the large muscle tears and haematomata at postmortems. Moreover, the blood was diluted throughout the experiment by the intravenous infusion of fuchsin solution at the rate of 40 c.c./hr. leading to hydraemia. Under the given conditions haemoglobin percentages of 50–75 after 4–5 hr. infusion must in fact be considered as relatively high values.

There was no correlation between the degree of dye excretion and the gravity of the shock condition as indicated by the arterial blood pressure or the swelling of the crushed limb. This was not to be expected. Compensatory mechanisms, which may maintain the arterial blood pressure for a considerable time, render it difficult to assess the condition of shock at a given moment of the experiment. Neither is the swelling of the crushed limb a true indication for increased permeation of plasma, since it results partly and to a varying degree from blood extravasates.

In only one experiment was the initial increased dye excretion after crushing absent. The procedure in this experiment differed from that adopted in the other twelve by the fact that the crushing was not limited to the parts distal to the knee joint but included the thigh. Post-mortem, a large haematoma was found round the femoral vessels probably compressing them. Some of the blood vessels to and from the knee joint were probably severed and nervous lesions could not be excluded. These facts may be responsible for the absence of the initial increased dye excretion.

In six experiments a colloidal dye, trypan blue, was infused in a 1 % concentration into the jugular vein simultaneously with the non-colloidal acid fuchsin. This dye, which does not pass the articular barrier under normal conditions [Engel, 1940], also failed to appear in the perfusate from the traumatized side, though it has the greatest degree of dispersity in the series of those dyes which do not pass the barrier of the normal knee joint. This may be due to the fact that the trypan blue is too toxic to be given in a sufficiently strong concentration or that the dye is taken up too readily by the reticulocytes.

Measurements of temperature of the quadriceps muscles showed no definite correlation between increased dye excretion and changes in temperature. Although in the majority of the experiments the temperature of the traumatized side was 0·3–1·0° C. higher than the control side the ratio was reversed in three. In one of these the temperature of t⌐   ᵗⁱᵐᵉᵈ ˢⁱᵈᵉ ˢᵗᵃʳᵗᵉᵈ ᵗᵒ rise

at the late stage of the experiment when the dye excretion had decreased and the temperature became even higher than that of the control side. In the experiment of Fig. 2 the temperature of both muscles throughout the experiment is given in the tracings at *A*.

Late effects

The late decrease in the excretion of the dye from the crushed leg was studied separately in twelve cats by crushing the leg 3–23 hr. before the perfusion of the knee joints was started. In ten of these the crushing was inflicted upon the calf and foot. Perfusion was started in eight. after 3–4½ hr., in two, after 23 hr. In only two experiments in which the perfusion was started 3 and 4 hr. respectively after the trauma was there an increased dye excretion from the joint of the traumatized limb. This increase was small (15 and 60% respectively) and lasted for 1 and 2 hr. only. Later on, excretion from the traumatized leg diminished and became considerably smaller than from the control side. In the other eight experiments the dye excretion was much smaller on the traumatized side from the beginning to the end of the perfusion (3–4 hr.). The difference amounted to between 100 and 600%. The increased dye excretion from the traumatized side observed in the two experiments must be regarded as a delay of the transition of the first phase into the second, indicating that the decreased excretion does not always begin exactly 3 hr. after the trauma but may be delayed for another hour or two. Six hours, however, was the maximum time limit within which increased dye excretion has been observed from the traumatized side. A typical experiment illustrating the late decrease in dye excretion from a traumatized limb is given in Fig. 3. Perfusion was started 23 hr. after crushing of the left leg, at a time when the animal was in a weakened general condition and when the left leg was considerably swollen. At the end of the experiment it was found to weigh 130 g. more than the right leg. In this experiment the early dye excretion from the control knee fell at about 3.30 p.m. simultaneously with the appearance of signs of heart failure. Shortly before death the excretion rose again, an effect repeatedly observed in a dying animal.

In two experiments the crushing was inflicted on the thigh and perfusion started 3½ hr. later. In the one experiment dye excretion from the traumatized side was 20% greater throughout the whole 2½ hr. perfusion than from the control leg. In the other experiment this condition was observed in the first half-hour, afterwards the excretion from the traumatized side decreased considerably and became much smaller than that from the normal side.

In five experiments trypan blue was infused simultaneously with the acid fuchsin. It did not appear in any sample of perfusate of either knee joint.

The temperature of the quadriceps muscle of the traumatized side was in most experiments 0·2–1·5° C. higher despite the much smaller dye excretion from the knee joint of this side.
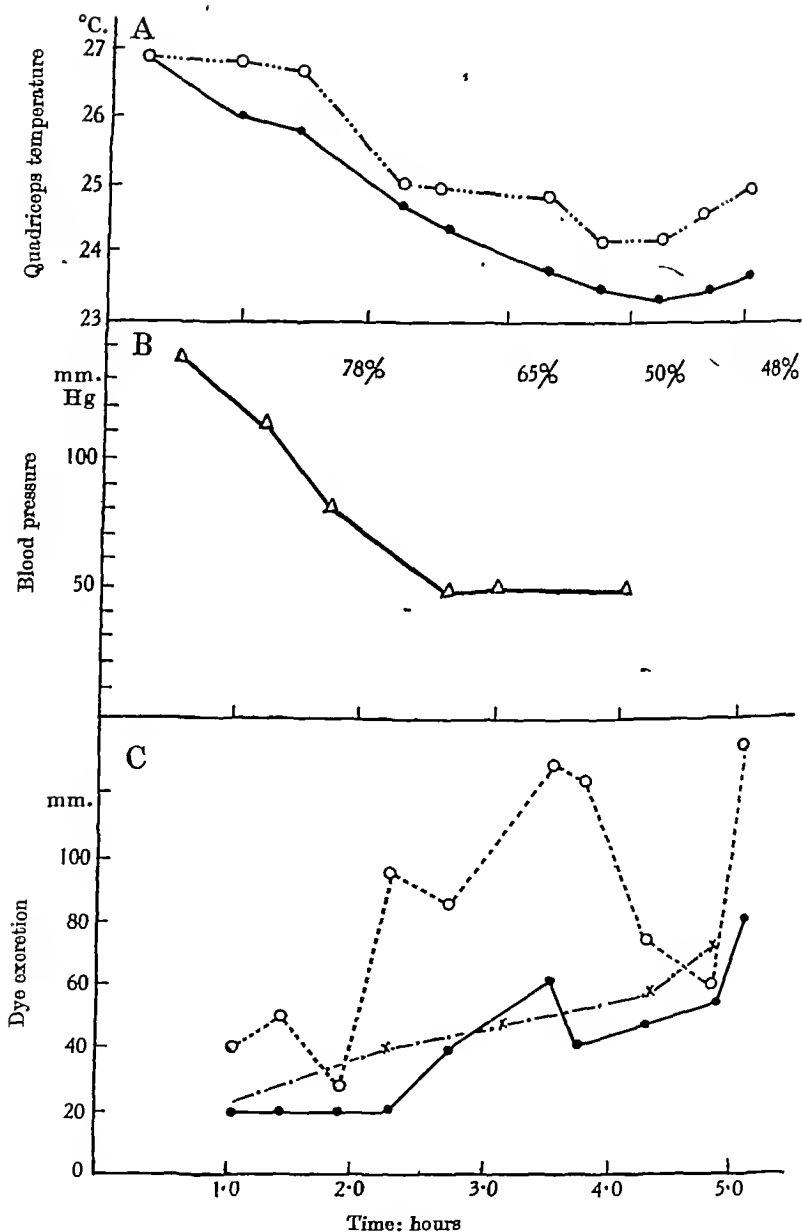
Fig. 3. Decreased dye excretion of traumatized right leg (continuous line) as compared with the excretion of the left control leg (dotted line), 23 hr. after trauma. Symbols as in Fig. 2.

Crushing both forelegs

Shortly after crushing both forelegs the dye excretion drops considerably from both knee joints in contrast to the rise in those experiments in which the trauma was inflicted near the perfusion area. A typical experiment is illustrated in Fig. 4. The dye concentration in this case fell from 24 to 5 mm. on the right and from 36 to 4·5 mm. on the left side to rise later to 13 and 10 mm. respectively. Such a secondary rise usually occurred 2–3 hr. after the trauma;
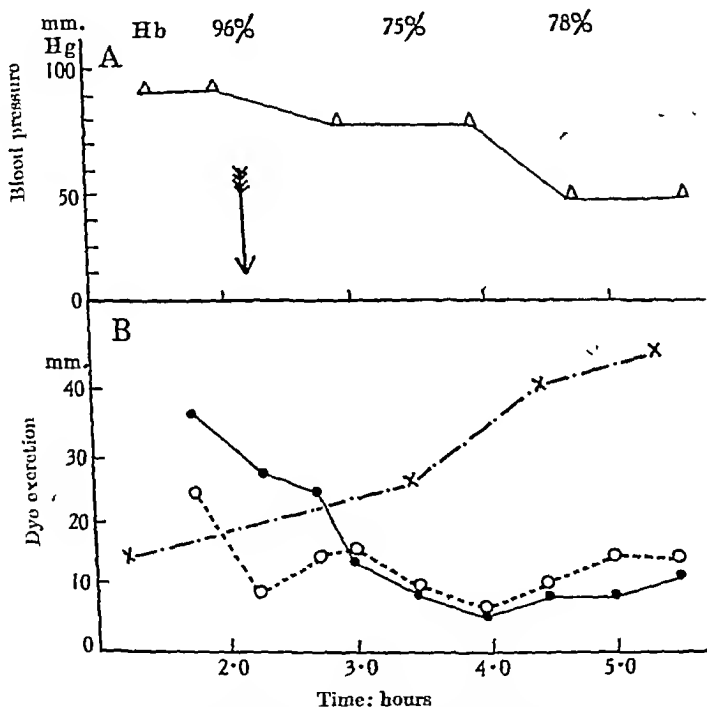
Fig. 4. Effect of crushing both forelegs on blood pressure (A), and dye excretion of both normal knee joints (B). Symbols as in Fig. 2.

sometimes the excretion reached the initial level or even exceeded it. However, in the meantime the dye concentration in the blood had also risen considerably. The secondary rise resembles the gradual increase in dye excretion which occurs in uninjured animals, perfused under otherwise similar conditions. It is due to the gradually increasing blood-dye level. This experiment was repeated eleven times with similar results. No immediate rise of dye excretion could be detected in the perfusates. Only one case deviated from this rule.

DISCUSSION

The fact that the dye excretion from the perfused knee joint first increases and later decreases if a trauma is inflicted on the same limb below the knee, but shows an immediate diminution if the trauma is inflicted on a distant part of the body, is difficult to explain fully with the data at our disposal. The initial increase in dye excretion could not have been due to the rising concentration of dye in the blood, since this would have affected both the normal and the traumatized side. The lack of parallelism between dye excretion from the knee joint and change in temperature in the quadriceps muscle of the same leg makes it unlikely that the increased excretion resulted from a better blood supply to the traumatized leg, or that the subsequent decreased excretion had resulted from a reduced blood supply. The supposition was that the quadriceps muscle as the most intimately related to the knee joint has a very similar blood supply and will show a reaction similar to the joint. Though this assumption may be open to criticism, the fact that the temperature changes sometimes were in direct opposition to those to be expected if the dye excretion had been dependent upon the blood supply, supports the view that the observed changes in dye excretion which occurred when the trauma was inflicted on the same leg, are not, or at least not mainly, the outcome of changes in blood supply but of changes in the rate of filtration through the capillaries.

The immediate increase in rate of filtration through the capillaries in the neighbourhood of the trauma may be interpreted in different ways: one possibility, and in our opinion the most probable one, is that the capillary permeability in and around the area of trauma is temporarily increased. It is known [Landis, 1934] that the movement of fluids through the capillary wall is greatly influenced by the capillary blood pressure, the colloid osmotic pressure of the plasma proteins and the retention of proteins by the capillary wall. These factors were not measured in our experiments and therefore the theory of increased permeability as a cause of the increased dye filtration is not fully conclusive.

Another possible explanation is that the increased dye excretion is not a sign of an increase in general permeability of the capillaries in the knee joint, but that the acid dye may pass more readily through the membrane after the trauma because of a lowering of the pH.

It is unlikely that the late decrease in dye excretion which follows the period of increased dye excretion, when the trauma has been inflicted near the perfused region, is due to the same mechanism as that responsible for the immediate decrease in filtration through the capillaries, when the trauma has been inflicted at a distant part of the body, since the one condition develops when the other disappears. The immediate and late changes in permeability when the trauma is inflicted near the perfused joint closely resemble the conditions

developing in inflammation. With the same technique as used in our experiments it has been found [Engel, 1940] that the permeability in the knee joint, 24 hr. after an intra-articular injection of a 1% Lugol solution, was decreased as compared with the normal side, while according to Tani [1935], who used a different technique, dye excretion was increased during the first hours of inflammation caused by Lugol solution. In the area adjacent to the trauma a similar sterile inflammation appears to develop. This condition resembles or may be identical with what Eppinger, Faltitschek, Kaunitz & Popper [1935] described and demonstrated histologically as a serous inflammation. In the course of it the space between the synovial membrane and capillary endothelium would be imbued with fluid, probably plasma, and the double layer barrier changed into a more composite one which would offer greater resistance to the penetrating substances.

The immediate decrease in dye excretion from the knee joint when the trauma had been inflicted on the forelegs is most probably the result of a fall of dye concentration in the circulating blood, brought about by loss of dye into the traumatized tissue of the swollen forelegs. There is no fall of blood dye concentration in curve *B* in Fig. 4, possibly because it was missed between the two readings at 3.30 and 4.30 p.m. In other experiments the fall amounted to 30% and rose later again to the original level. A decreased dye concentration of the blood would lead to a fall of dye concentration in the articular perfusate without any change of the filtering capacity of the capillaries. This view is supported by the fact that this decrease in dye excretion lasts for a period of 3 hr., a time corresponding to the period of increased dye excretion into the traumatized tissue, as indicated by the experiments in which the trauma had been inflicted near the perfused knee joint.

Whatever the mechanisms responsible for the changes in dye excretion and in filtration through the capillaries may be, our results show that crushing tissue to such an extent that death ultimately ensues, does not cause an increase in filtration through the capillaries throughout the body, but only in the neighbourhood of the traumatized tissue. Thus our results support the view expressed by Moon [1938] that 'the main factor which is most probably responsible for the continued low blood pressure after injuries is not a general increase in capillary permeability with loss of fluid all over the body, but a loss of blood plasma through the walls of the damaged capillaries'. The conception of Short [1913], on the other hand, that 'oligaemia in shock is induced by loss of fluid partly into the injured area, partly through the capillaries all over the body' is not borne out by our experiments.

The time limitation of increased permeation in the region adjacent to the trauma, and probably also at the site of the trauma, may have a practical significance for the treatment of cases of traumatic shock. Any therapeutic action intended to arrest the outflow of plasma from the circulation ought to be done

within the first 3–5 hr. after the trauma, since after that time the natural development tends to arrest this condition without outside interference. This conclusion bears out the experience of Slome & O'Shaughnessy [1938] that 'the subject of severe trauma who does not show some sign of recovery under established modes of treatment within two or three hours of his injury is almost inevitably doomed'. The sealing up of the portals from the blood vessels to the tissues may be of mixed benefit, since it does not only stop the outflow of plasma into the tissue, but renders also the reflow into the reversed direction more difficult. This is probably one of the reasons why shock, after having reached a certain stage, is an irreversible condition.

It might be argued that the knee cavity with its synovial membrane is not a true representative of the limb tissue and that the observed changes in dye excretion, as a result of changes in permeability, do not necessitate corresponding changes of permeability in other vessels of the limb. Since the joint cavity, however, is embryologically a natural slit in the limb, and the synovial membrane a derivate of conjunctive tissue, since further its blood supply and innervation are closely related to those of its surroundings, it appears reasonable to assume that it participates equally in all physiological and pathological processes.

SUMMARY

1. When the hind leg of a cat is crushed below the knee and dye (acid fuchsin) injected intravenously, its excretion from the perfused cavity of the knee joint of the same leg increases during the first 1–5 hr. and decreases afterwards. These results suggest an initial increase followed by a late decrease in filtration through the capillaries in tissue adjacent to the trauma. An increased rate of filtration under conditions otherwise unchanged would indicate an increased permeability of the capillaries.

2. There is no corresponding increase in dye excretion from the perfused knee joint when the crushing is inflicted on the forelegs. In this condition the dye excretion decreases during the first 3 hr., probably as a result of a fall in dye concentration in the blood, caused by loss of dye into the traumatized tissue of the forelegs.

3. These results support the view that in 'traumatic shock' increased filtration through the capillaries does not occur throughout the body, but is restricted to the tissue in the neighbourhood of the injury; it occurs there only during the first few hours after the trauma.

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THE EFFECT OF EXERCISE ON CHLORIDE EXCRETION IN MAN DURING WATER DIURESIS AND DURING TEA DIURESIS

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In studies on the effect of exercise on urinary excretion in man, some diuretic is habitually used to facilitate frequent sampling of urine. Water has been most usually employed in this connexion, but tea was used in one major work on the subject [MacKeith, Pembrey, Spurrell, Warner & Westlake, 1923]. Comparison of results obtained under the action of these two different diuretics is unreliable, owing to the different conditions prevailing in the different sets of experiments, especially in regard to the severity and duration of the exercise undertaken. An attempt has now been made to differentiate the changes in urinary excretion due to the exercise and those due to the diuretic employed, by experiments under more comparable conditions.

METHODS

Students of both sexes were used as subjects, details of whom are given in the appendix. The last meal was taken 5–6 hr. previously, and a glass of water $2\frac{1}{2}$–3 hr. before the experiment. The exercise was short and severe—an 'all-out' sprint occupying 40–60 sec.

The first comparison was made, in co-operation with the Biochemistry Department, between two different groups of subjects, at one year's interval. On the first occasion, small amounts of water were taken repeatedly in order to promote a reasonable flow of urine, and on the second occasion, a pint of tea was taken in one draught. The beverage was made by extracting $\frac{1}{2}$ oz. Indian tea with boiling water for 5 min. and contained less than 20 mg. NaCl/100 c.c. The exercise followed when diuresis was well established, two pre-exercise and four post-exercise urine samples being collected for subsequent analysis by the rest of the class. In the remaining experiments a more accurate comparison was made by using each subject for both experiments. These were done at the same time of day (afternoon); 1 pt. of fluid (either water or tea) was imbibed within 2–5 min. and the exercise taken 30–40 min. later when diuresis was well

established. Urine sampling was more frequent than in the class experiments, but chloride only was analysed (electrometric titration method [Eggleton, Eggleton & Hamilton, 1937]).

RESULTS

Class experiments

The main results of the class experiment on the effect of exercise on urinary excretion during water diuresis have been published already [Eggleton, 1942 *a*]. Their interest at the moment lies in the comparison with them of the results
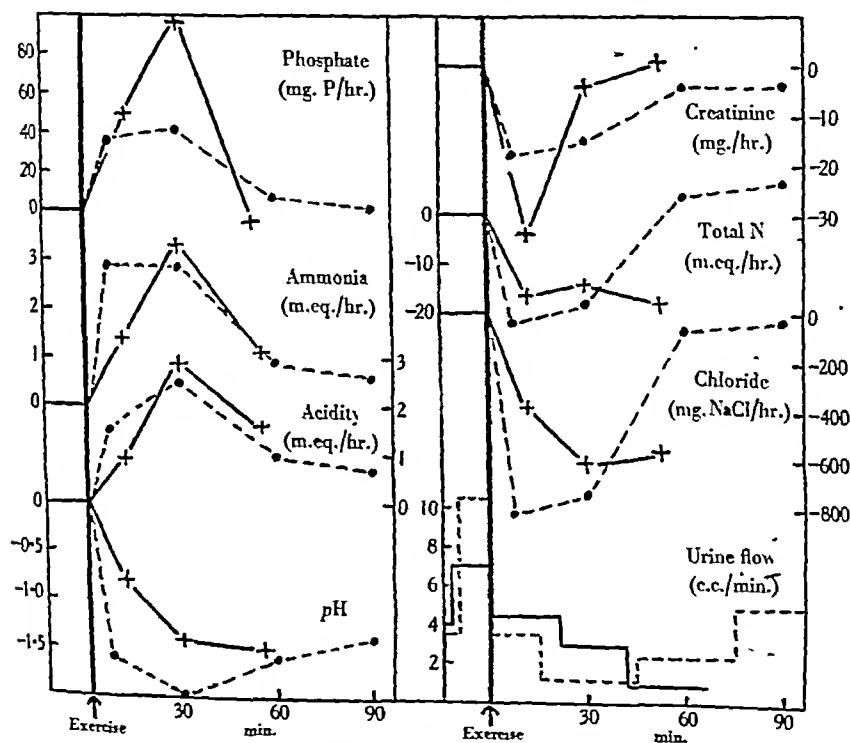
Fig. 1. Average changes in urinary excretion following exercise during tea and during water diuresis. +——+ Water diuresis (average of seven subjects). ●----● Tea diuresis (average of five subjects).

obtained subsequently during a tea diuresis. Such a comparison of the average results (calculated as changes on a pre-exercise value of zero) is shown in Fig. 1.

It is clear that most of the changes observed are common to both experiments and can, therefore, be attributed to the exercise as such. This results in an increase in the output of phosphate, of titratable acidity and ammonia, and a fall in pH; and a temporary reduction in the output of creatinine, chloride and total nitrogen. Minor differences between the two sets of results, either in time lag or in magnitude, can be readily explained by slight differences in technique

on the two occasions. In the water-diuresis experiment, for example, some minutes elapsed between the collection of the last pre-exercise sample and performance of the exercise; the first post-exercise sample was, therefore, a mixed one, and changes in composition due to the exercise were thus artificially reduced. In the tea-diuresis experiment the exercise was rather less severe and of longer duration (90 sec. instead of 50–60) owing to a misunderstanding of instructions, and the increase in phosphate output was therefore less. The apparent difference in depression of creatinine excretion is, however, barely significant, this analysis being the least reliable of any in student hands.

The only differences of any significance between the two sets of experiments lie in the excretion of chloride, of total nitrogen, and of water. During the tea diuresis both total nitrogen and chloride excretion return to their pre-exercise level within an hour, but during the water diuresis they do not. This latter result confirms the observation of Havard & Reay [1937] in regard to chloride excretion. The experiment was, however, terminated sooner than that performed during tea diuresis, and the results therefore do not negative the possibility of a later return to normal values.

In order to determine whether the exercise or the diuretic employed was responsible for the observed differences in the results of these two class experiments, a more careful comparison was made on a group of individuals, attention being focused on chloride excretion and water output. For this purpose, eleven subjects were used, each performing the same amount of exercise on two succeeding afternoons; on one day a pint of water was taken first and on the other a pint of tea. In nine of these subjects, control experiments on tea and water diureses were made, without exercise.

Chloride excretion in water diuresis

In eight of the nine subjects tested, water diuresis itself led to a considerable diminution in chloride output. Curves of the average rate of urine flow and chloride excretion for these eight subjects are shown in Fig. 2. It is strange that this effect accompanying a water diuresis has not previously been emphasized, for evidence of its existence is not lacking in the literature. Marshall [1920], for example, who was attempting to demonstrate a rise in chloride excretion during water diuresis in man had to admit 'at the height of urine flow, the elimination of chloride is diminished'. What he did not admit, but can be seen clearly in his protocols, is a still greater decrease in chloride excretion as the diuresis continued; this occurred in all the subjects investigated. Priestley [1921] and Adolph & Ericsen [1926] obtained a similar result.

A study of individual curves shows no close time relationship between the decrease in chloride output and increase in water excretion. In Fig. 3 two extreme cases are plotted; in one (subject G), the greatest fall in chloride excretion precedes the main onset of diuresis, and in the other (subject L), the

lowest point on the curve is reached only as the diuresis is passing off. This fact suggests the possibility that two distinct mechanisms may be involved, the relative sensitivity of the two varying in different individuals. A comparison of the total output of water and of chloride in the different subjects, however, suggests the existence of an inverse relationship between the two. In Table 1 is shown the total output of urine in the eight subjects, in descending order of
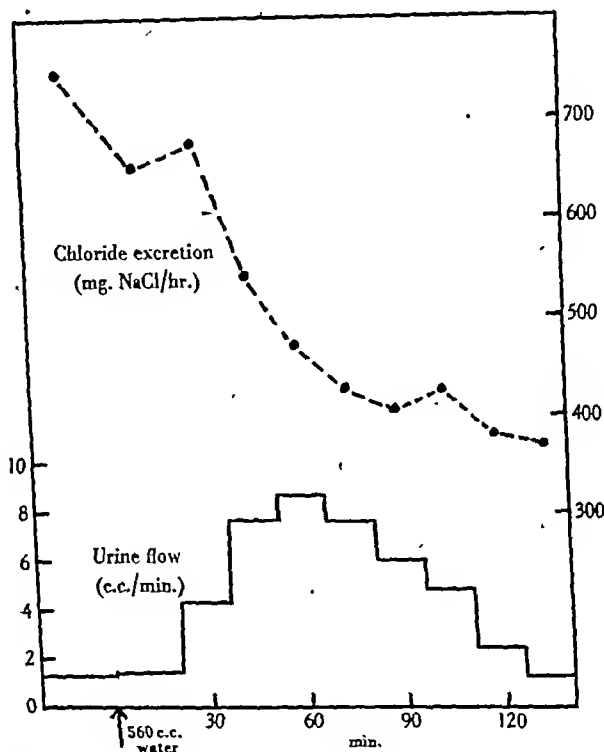
Fig. 2. The effect of a water diuresis on chloride excretion. Average of eight subjects.

magnitude, together with the decrease in total chloride excretion expressed as a percentage change on the normal resting output. Although the relationship is not a close one, there is a definite trend in the direction of greater inhibition of chloride excretion with smaller diuresis.

In one of the nine subjects investigated, a completely different result was obtained. The chloride excretion increased steadily with increase in rate of urine flow during the onset of the diuresis, as can be seen in Fig. 4. The same phenomenon was observed in the exercise experiment, during the onset of diuresis before the exercise, and would appear, therefore, to be of regular occurrence in this individual. On the two occasions on which the resting rate of flow was measured, it was considerably lower than that encountered in any

other subject: 0·28 and 0·36 c.c./min. in contrast with a minimum value of 0·65 c.c./min. in any other. The subject is apparently quite normal and healthy, and unfortunately opportunity has been lacking for any further investigation which might throw light on this abnormality.

Fig. 3. Showing the lack of close relationship between the course of the decrease in chloride excretion (right ordinate) and increase in rate of urine flow (left ordinate) during water diuresis in two different subjects.

TABLE 1. Comparison of the response of different subjects to 560 c.c. water

Subject	Total urine output in 2½ hr. c.c.	Reduction in total chloride output %	Subject	Total urine output in 2½ hr. c.c.	Reduction in total chloride output %
D	885	10	G	675	42
C	795	21	M	660	39
L	730	16	F	530	50
H	705	20	B	370	41

Chloride excretion in tea diuresis

Comparison of water and tea diureses in the same individuals did not yield as much information as had been anticipated. The tea was not tolerated well (the same brand as that used earlier could no longer be obtained), and in many cases the stomach emptied irregularly, or in part by way of the oesophagus. Thus a comparison of total output of water in the two forms of diuresis was possible in only five subjects. In these, the tea diuresis was consistently greater than the water diuresis, on the average 35% greater.
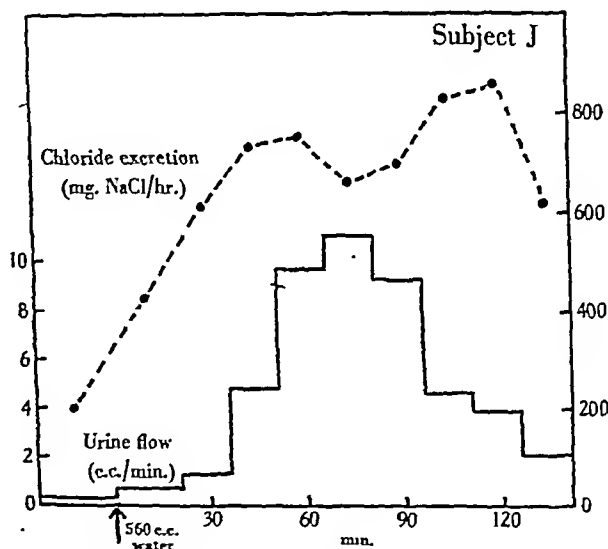
Fig. 4. Abnormal behaviour of chloride excretion during water diuresis in one individual.

The effect of the tea on chloride excretion was even greater. In spite of the fact that the chloride excretion should have been depressed by the volume of water in which the tea was taken, it was in fact increased in all cases, even when the water output was depressed owing to nausea. The average change in rate of chloride excretion for all subjects except J (in whom no normal resting chloride value could reasonably be assessed), expressed as a percentage of the normal resting value, was 150% in tea diuresis and 70% in water diuresis. The results plotted in Fig. 5 give some idea of the magnitude of the opposing actions of water and of tea on chloride excretion when the diuresis is uninterrupted. The resting value of 1·0–1·2 g. NaCl/hr. (the experiments were performed at 1 week's interval) rose to 1·8 g./hr. under the action of tea and fell to 0·4 g./hr. under the action of water.

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L	730	16	F	530	50
H	705	20	B	370	41

abnormal in showing practically no inhibition of urine flow. He returned in 1942 for the purpose of this experiment and his curve (E) in Fig. 6 closely resembles that obtained in 1941; in 1941, however, less inhibition was observed in the first $\frac{1}{4}$ hr. after the exercise. The curves in Fig. 6 are arranged in order of increasing duration of inhibition. In the penultimate one (average of subjects G and K), the inhibition immediately following the exercise is separated from the later stage by a temporary return of diuresis. In the last curve (D),
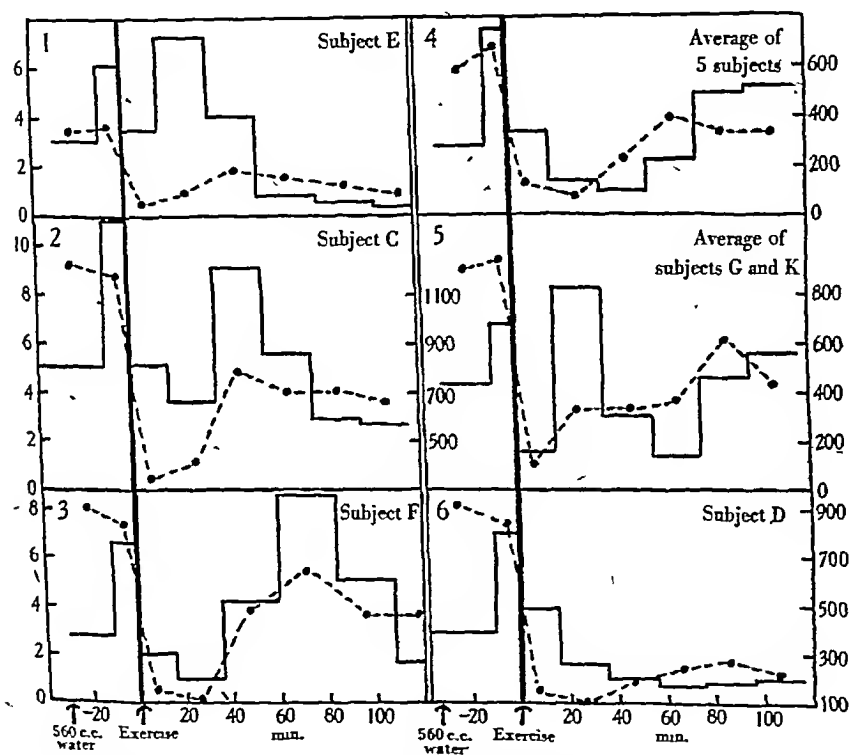
Fig. 6. The effect of strenuous exercise on the course of a water diuresis in different individuals.
—— Rate of urine flow (c.c./min.). •·····• Chloride excretion (mg. NaCl/hr.).

diuresis had shown no sign of returning by the end of the experiment. The possible and probable causes of this inhibition, and of the changes in output of chloride, will be considered in the discussion.

In spite of the inhibition of urine flow following the exercise, no consistent relationship was observed between the total output of water with and without exercise over the period studied. Subject D, for example, who gave one of the smallest responses after exercise (330 c.c. in $2\frac{1}{2}$ hr.), showed the largest water diuresis (885 c.c./$2\frac{1}{2}$ hr.) without exercise. In contrast with this result, three subjects (C, F and G) actually showed a larger total output of water in the exercise

The actual course of the chloride excretion varied widely in different subjects, as in the water-diuresis experiments, even when the stomach appeared to empty normally. The variation in urine output among the few successful experiments was, however, too small to permit any deductions to be made from a comparison of total water and total chloride output, as was done in the case of water diuresis (Table 1).
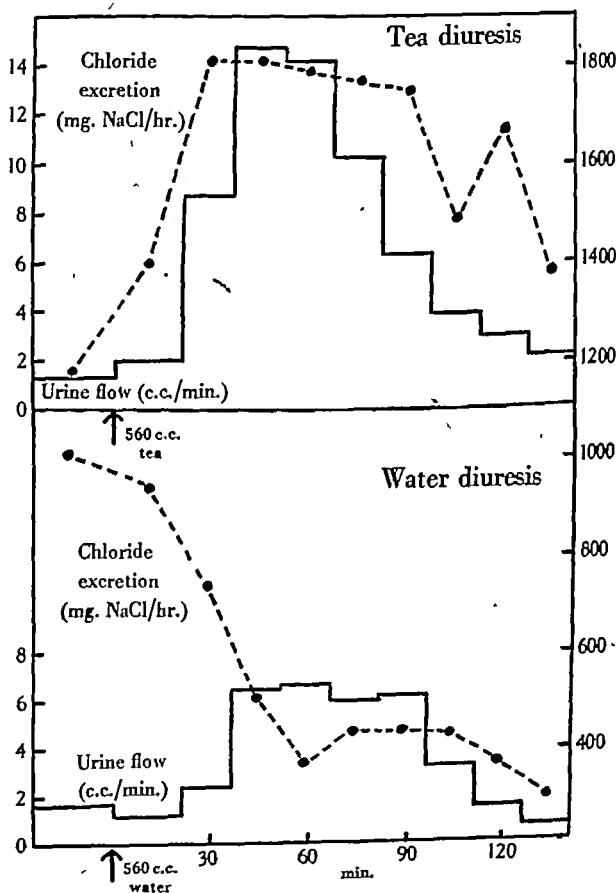
Fig. 5. Showing the opposing actions of water and of tea on the chloride excretion in one subject (F).

Individual variation in the course of water diuresis following exercise

In Fig. 6 is illustrated the wide range of individual response to exercise encountered during the water diuresis. If this occurred in the class experiment of 1941, it passed unnoticed, possibly owing to the fact that sampling was continued for only 60 min. after the exercise and that the water was taken in divided small doses; one of the eight subjects used then was considered

appreciably decreased as a result of the exercise, in one it was unchanged and in the fourth increased; and the chloride excretion was increased in two, and decreased in two.
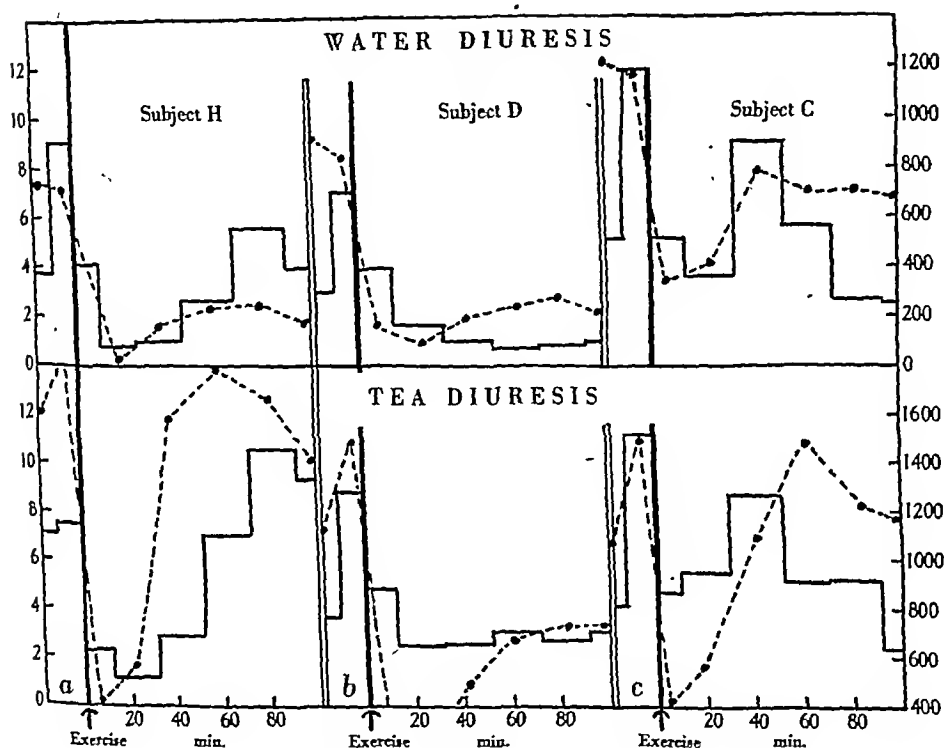
Fig. 7. Comparison of the effect of strenuous exercise on the course of a water and a tea diuresis in three different subjects. ——— Rate of urine flow (c.c./min.). •----• Chloride excretion (mg. NaCl/hr.).

TABLE 3. The total output of chloride during tea diuresis and during water diuresis with exercise

Subject	Total chloride output in 2·5 hr. g. NaCl	
	Tea diuresis	Water diuresis
A	2·27	1·75
B	2·09	0·5
C	2·88	2·02
D	1·87	1·0
E	1·87	0·46
F	3·2	1·33
H	3·7	0·8
J	2·99	0·7
Average	2·61	1·07

experiment than in the uninterrupted water diuresis. Comparison of chloride excretion under the same two sets of conditions yields a similar negative result. The total chloride output was reduced on the average to 70% of its normal value by the water diuresis, and to 62% of the normal value as the result of combined water diuresis and exercise, but owing to the wide individual variation this small difference has no significance.

Comparison of tea and water diureses (with exercise)

Only eight successful experiments were made with tea diuresis, nausea or vomiting having occurred in the remaining three. In all eight cases, the total output of urine in the $2\frac{1}{2}$ hr. following the drink was greater than in the water-diuresis experiments. These figures are shown in Table 2. The relative constancy

TABLE 2. The relative magnitude of tea and of water diuresis with exercise in different individuals

	Urine output in 2·5 hr. c.c.		Ratio of tea/
Subject	Tea diuresis	Water diuresis	water diuresis
A	825	525	1·57
B	477	325	1·47
C	876	838	1·05
D	552	330	1·67
E	924	472	1·96
F	953	672	1·41
H	965	585	1·65
J	660	406	1·62
		Average	1·55

of the ratio of the two suggests that the large variations observed in the total output of different individuals is not a haphazard effect, but due to some specific difference in their reaction to exercise and the diuretic combined. This impression is enhanced when individual curves of tea diuresis and of water diuresis are compared. Three different types of response are shown in Fig. 7, and the same parallelism in response to the two diuretics was shown in the remaining five subjects. As in the water-diuresis experiments, there is little apparent relation to be seen in the tea diuresis between the output of chloride and the course of diuresis. The total chloride output is consistently higher than that following a water diuresis, as can be seen from the figures in Table 3. In the pre-exercise period it increases markedly with the onset of diuresis (contrary to its behaviour in water diuresis), and is already raised above normal in the first sample (Fig. 7).

The few comparisons available between experiments under tea diuresis, with and without exercise, yield results similar to those obtained under water diuresis. No regular relationship was found in any individual, either in regard to total output of water or of chloride. In two subjects, the urine output was

of urine flow which can be attributed to release of anti-diuretic hormone. It is likely, therefore, that this factor operates in any type of exercise, and was probably the only one concerned in the experiments of MacKeith *et al.* [1923] on man; they observed a temporary reduction in rate of urine flow during a tea diuresis whenever a walk gave place to a trot. In subject E no further explanation is required. In the remaining subjects, a further period of inhibition lasting from 20 to 60 min. precedes the return of diuresis, and in two (G and K) this prolonged period is separated from the initial period of inhibition by a short-lived return of diuresis.

It is unlikely that delayed absorption can account for this large individual variation. True, there is little accurate evidence as to the rate of absorption of water in man. In dogs weighing 10 kg., Klisiecki, Pickford, Rothschild & Verney [1932] found 250 c.c. absorbed in 35 min. on the average. From less direct measurements in man, Smirk [1933] concluded that absorption was complete (1000 c.c. in 27–55 min., and 500 c.c. in 22 min.) before maximum diuresis was attained. In our experiments, not less than 30 min. elapsed between the drink and the exercise, and in one or two cases where full diuresis had not set in by this time, the exercise was delayed a further 10–15 min.

The only factor peculiar to the particular type of exercise employed in these experiments (short and strenuous), apart from the temporary vasoconstriction, is the undoubted concentration of the blood which follows. Gregersen, Dill & Meade [unpublished experiment quoted by Gregersen, 1938] demonstrated a 12–16% concentration of the blood (haemoglobin, plasma dye, and plasma proteins) as a result of 90 sec. intensive running, and computed that 500 c.c. water had left the blood stream in the next 90 sec. The *status quo* was restored fully only 1 hr. later. Chloride does not pass readily into muscle fibres, and it is likely that plasma chloride is concentrated to the same degree as other constituents in these circumstances. This concentration of electrolytes will lead to an active secretion of anti-diuretic substance. It seems not improbable that the degree of plasma concentration and its duration might well vary in different subjects, with consequent variation in the secretion of anti-diuretic hormone, both in amount and duration, sufficient to account for the variable prolonged inhibition of diuresis observed in our experiments. In addition, the possibility remains that the 'pituitary mechanism' is more readily stimulated in some individuals than in others, a possibility suggested by previous work on a comparison of water and alcohol diureses [Eggleton, 1942b]. The relative constancy of the duration of this inhibition in any one subject under tea and water diureses (Fig. 7) would support equally either hypothesis. Viewing the evidence as a whole, it seems justifiable to conclude that the most likely cause of the prolonged inhibition of urine flow following the exercise is secretion of the anti-diuretic hormone.

To what extent the changes in chloride excretion may also be explained on

DISCUSSION

It must be admitted that the original point of the research—the effect of exercise on chloride excretion—has not been fully cleared, owing to the opposing actions of the diuretics used. A comparison of the course of chloride excretion with and without exercise, however, indicates the probability that changes occurring in the 40–50 min. after exercise are the only ones which have any significance in relation to the exercise in the majority of subjects. The immediate decrease may be attributed, as also the decrease in water output, to a temporary vasoconstriction. This is indicated by the sharp decrease in creatinine excretion observed during the first 15 min. after the exercise in both class experiments, and is seen only after very strenuous exercise. In the experiments of Wilson, Long, Thompson & Thurlow [1925] on man, for example, rather less severe exercise (1–2 min. stair-running) during a water diuresis resulted in a prolonged inhibition of chloride excretion (40–60 min.) and of urine flow (60–90 min.), but had no effect on creatinine excretion.

The continued decrease of chloride excretion in the next period, observed under both tea and water diureses, coincides with, and may possibly be related to, the peak excretion of acid products of muscular activity. The factors controlling electrolyte excretion are still obscure, but there seems little doubt that if any one is greatly increased, others are diminished. Both increased sulphate and increased bicarbonate depress chloride excretion [Homer Smith, 1937] and the lactate ion may well have the same effect.

During the following hour, chloride excretion gradually rises again, there being no close correlation with increase in water output, and subsequently falls in the majority of subjects. These changes are much greater under tea diuresis than under water diuresis. The major part of the rise can be attributed to the reassertion of the caffeine effect and removal of the previous inhibiting effects (vasoconstriction and excretion of acid ions). The later fall is open to at least two interpretations: (a) a wearing off of the caffeine action, and/or (b) onset of the normal reduction accompanying the re-established diuresis. The latter explanation is favoured by the fact that it is most marked when a large diuresis has again set in, and by the similarity in the trend of the curve following water diuresis (see Fig. 6). When diuresis fails to reappear (Fig. 7b) this kink in the chloride-excretion curve is scarcely apparent irrespective of whether water or tea has been used as the diuretic agent.

The causes of the varying degrees of inhibition of water output after exercise in different individuals is again largely a matter of surmise. The decreased output during the first 15 min. may be attributed either entirely to vasoconstriction or in part also to a nervous interference with the pituitary mechanism. Rydin & Verney [1937] have shown clearly in dogs that mild exercise, or the anticipation thereof, during a water diuresis, produces a temporary inhibition

large bulk of water. It might well be suggested that the variation in response of different individuals was due merely to differences in their response to the water, the course of chloride excretion following ingestion of the tea being the resultant of increase due to tea extract and decrease due to the water. This simple explanation is, however, not substantiated by a detailed comparison of the course of chloride excretion in tea and water diureses in different individuals.

The experiments as a whole throw some further light on the problem of individual variation in response. It has been demonstrated previously [Eggleton, 1942 b] that a large individual variation exists in the diuretic response to water, and it was expected that these individual variations would persist when the effect of exercise was superimposed. This, however, was not observed. Subject D, for example, who showed the largest diuretic response to water alone, gave one of the smallest responses to water with exercise. Variations in response to exercise are presumably masking variations in response to the diuretic. Experiments on tea diuresis fully confirm this conclusion. Individual variations have been shown to occur in response to tea, which are correlated with those in response to water, the average diuretic response to the former being 1·35 times that to the latter. As in the case of water diuresis, however, individual variation in response to exercise overrides that in response to tea. When exercise is superimposed on both diureses, the correlation between the response to water and the response to tea is once more apparent, and, in any one subject, the effect of the exercise is the same whether diuresis is produced by tea or by water (Table 2).

SUMMARY

1. A short period of severe exercise superimposed on a tea diuresis results, as in a water diuresis, in an increased excretion of phosphate, titratable acidity and ammonia, and a fall in pH. A temporary decrease in output of creatinine, total nitrogen and chloride also occurs under both sets of conditions (Fig. 1).

2. The more permanent decrease in total nitrogen, chloride and water output following exercise during water diuresis is not observed during tea diuresis.

3. A more accurate comparison of the effects of exercise on the output of water and chloride during the two types of diuresis, made on the same individuals, gave the following results:

(a) A large individual variation occurred in the duration of inhibition of water diuresis as the result of such exercise (Fig. 6).

(b) This variation was not haphazard; the same duration of inhibition was observed also under tea diuresis in the same individual (Fig. 7).

(c) The total output of water also varied widely in different individuals. That following tea diuresis was always greater and bore a fairly regular relation to that following water diuresis (1·55 times as great) (Table 2).

this basis is a more open question. If lack of anti-diuretic hormone is responsible for the decrease in chloride excretion observed in a water diuresis, the temporary increase in excretion of this ion during the prolonged inhibition of urine flow following the exercise might be attributed to reappearance of the hormone in circulation. The lack of any close correlation between the increased water output and decreased chloride excretion, however, either in an uninterrupted water diuresis (Fig. 3) or following exercise (Fig. 6), does not lend much support to this view. Nor have other workers succeeded in demonstrating any increase in chloride excretion as a result of injected pituitrin unless massive doses of the hormone are used [Stehle, 1926; Corey & Britton, 1941]. Without further evidence, no certain conclusion as to the mechanism responsible for the suppression of chloride excretion during a water diuresis can be drawn.

The inverse relationship between magnitude of water diuresis and of decrease in chloride excretion suggested by the results presented in Table 1 does not necessarily lend support to either view. There is a considerable body of evidence in favour of the generally accepted belief that chloride excretion is increased with increased rate of urine flow owing to the faster passage of fluid through the tubules with consequent reduced opportunity for chloride reabsorption. In the results presented here, there are several hints that this factor is in operation in water diuresis, in opposition to the specific chloride-inhibiting action also present. In the curve of the average results (Fig. 2), for example, the decrease in chloride excretion in the earlier stages of the diuresis is temporarily checked with the first sharp rise in rate of urine flow, and in subject L (Fig. 3) this effect is very marked: similar results were obtained by Priestley [1921]. The inverse relationship suggested in Table 1 could be explained on this basis, the lack of any closer relationship being attributable to the varying strength of the opposing chloride-inhibiting factor in different individuals. In subject J (Fig. 4) one must assume a complete absence of this latter factor.

In the tea-diuresis experiments, these changes accompanying a water diuresis are masked by the opposing action of the tea extract in increasing chloride excretion. In total effect this action more than counteracts the inhibitory effect of the water on chloride output, so that the pint of tea causes a total output of chloride well above the normal for any individual. We have obtained no clear-cut evidence as to whether this increased chloride excretion is due to an increased glomerular filtration rate or to a specific action on chloride output, though the lack of correlation between height of diuresis and magnitude of increase in chloride excretion (Figs. 5, 7) might be accepted in support of the latter hypothesis. This conclusion would be unaffected by acceptance of the more recent views of the action of caffeine [Goodman & Gilman, 1940]—that its main diuretic action is due to inhibition of water reabsorption rather than to increased glomerular filtration. Admittedly, interpretation of the results obtained is complicated by the fact that the tea extract was administered in a

MUSCULAR ACTIVITY AND CHOLINE ESTERASE

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It was reported recently that the serum choline esterase activity is raised by muscular exercise in man [Richter & Croft, 1942a]. This observation suggested that the high choline esterase activities that have been found clinically in thyrotoxicosis and in acute emotional states [Tod & Jones, 1937; Richter & Lee, 1942 a, b] might be due to the increased neuro-muscular activity commonly seen in these conditions.

The investigation has now been pursued with a view to defining more clearly the relationship between the serum choline esterase and muscular activity. The source of the additional choline esterase which appears in the serum during muscular effort has also been investigated.

METHODS

In the majority of experiments the authors served as subjects, but we are particularly indebted to Dr M. S. Jones and Dr R. Scarisbrick for giving us specimens of blood from a number of 'effort syndrome' patients and controls before and after exercise. The study of patients with 'effort syndrome' appeared relevant to the present investigation since in this condition the normal physiological responses to muscular effort (tachycardia, breathlessness, sweating and fatigue) are generally exaggerated; but the patients showed no essential difference from the controls as far as the choline esterase changes were concerned and the experiments on them entirely confirmed the experiments on normal individuals. Apart from the effort syndrome patients, all the experimental subjects were believed to be normal.

Venous blood specimens were taken from the cubital veins. Capillary blood specimens of 1·5–4·0 c.c. were obtained by expressing the blood from an incision made above the finger nail. Care was taken to avoid venous stasis while taking the blood.

Choline esterase estimation. Esterase activities were estimated by the manometric method with a Warburg apparatus under similar conditions to those described by Richter & Croft [1942b]. In carrying out the estimation 0·5 c.c. of

(d) Chloride excretion was greatly diminished for 35 min. after the exercise in both types of diuresis. The subsequent rise was much greater in the tea diuresis but fell again as water output increased. The total chloride output in water-diuresis experiments was only 40% of that in the tea-diuresis experiments (Table 3).

4. Control-diuresis experiments without exercise showed:

(a) A decrease in chloride output during water diuresis (Fig. 2), averaging 30%. The course of this change was not closely related to the increase in output of water (Fig. 3), and in absolute magnitude varied roughly inversely with the degree of diuresis (Table 1).

(b) An increase in chloride output during tea diuresis (Fig. 5), averaging 50%.

5. It is suggested that the inhibition of diuresis immediately following the exercise is due to vasoconstriction and nervous interference with the pituitary mechanism, and that the more variable and prolonged inhibition may be due to secretion of anti-diuretic hormone resulting from the concentration of blood resultant on the exercise.

APPENDIX

Subject	Sex	Body wt. kg.	Subject	Sex	Body wt. kg.
A	♂	68	G	♂	66
B	♂	71·5	H	♂	58·5
C	♂	61	J	♂	63
D	♂	69	K	♂	65·5
E	♂	57·5	L	♂	51
F	♂	73·5	M	♂	75

All subjects were between 19 and 21 years of age.

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TABLE 1. Serum choline esterase changes with exercise

The subjects were all adult males 60–80 kg. in weight. Subjects P. 1, P. 2, P. 4 and P. 8 were effort syndrome patients. The 'press-ups' in Exp. 3 and Fig. 1 consisted of raising the body from the prone position by extending the arms. The bicycle ergometer was pedalled at a constant rate with a load adjusted so that the work performed was 6000–8000 ft. lb./min. (830–1100 kg.m./min.). Venous blood specimens were taken from the arm in the ergometer experiments; capillary blood was taken in the other experiments. The patients were taken from the Effort Syndrome Unit at Mill Hill Emergency Hospital and they were diagnosed by Dr M. S. Jones. In the experiments with patients oxalated plasma was used instead of serum.

Subject	Exercise	Duration min.	Total work kg.m.	Serum choline esterase μE./c.c./hr.	
D.R.	Raising 4 kg. wt. with right arm 20 times per min.	10	1,120	(a) Resting	252
				(b) After exercise	320
				(c) 10 min. after exercise	282
				(d) 15 min. after exercise	254
W.D.	Raising 4 kg. wt. with right arm 20 times per min.	2	167	(a) Resting	205
				(b) After exercise	212
				(c) 20 min. after exercise	205
D.R.	30 'press-ups'	5	5,760	(a) Resting	273
				(b) After exercise	306
				(c) 18 min. after exercise	268
P. 1	Bicycle ergometer	4	3,880	(a) Resting	126
				(b) After exercise	152
				(c) 20 min. after exercise	126
P. 2	Bicycle ergometer	5	4,850	(a) Resting	79
				(b) After exercise	100
P. 4	Bicycle ergometer	25	24,250	(a) Resting	90
				(b) After exercise	106
P. 8	Bicycle ergometer	5	4,850	(a) Resting	168
				(b) After exercise	183
				(c) 10 min. after exercise	168

a simultaneous fall in the choline esterase activity of the corpuscles (Table 2). Further, assuming the haematocrit to lie within normal limits, the fall in corpuscle esterase activity corresponded approximately in amount to the rise in the serum. This indicated that the extra choline esterase appearing in the serum as a result of muscular exercise most probably had come from the red blood corpuscles.

The results are not due to a partial haemolysis since haemolysis would not lead to a lowering of the concentration of choline esterase (μE./c.c./hr.) in the remaining red blood corpuscles. In any case the sera showed no trace of haemoglobin when carefully examined by eye, while haemolysis of less than 0.2 % of the red blood corpuscles could have been readily detected.

It was noticeable that in four of the subjects the loss of choline esterase from the red blood corpuscles continued in the 10 min. after the cessation of exercise.

Specificity of the choline esterase appearing in the serum after exercise. Human serum contains a choline esterase which hydrolyses methyl butyrate and tributyrin in addition to choline esters. The red blood corpuscles, on the other hand, contain two esterases, (a) a choline esterase which is highly specific for choline esters, and (b) an ali-esterase which is not inhibited by eserine and which

a 2·5 % solution of acetylcholine chloride in 0·9 % NaCl was put in the Warburg cup. The serum (0·2 c.c.) was diluted immediately before use to 4 c.c. with a solution containing 0·03 M $NaHCO_3$ and 0·12 M NaCl which was kept in a closed bottle in equilibrium with 5 % CO_2 in oxygen. The diluted serum was put in the main part of the Warburg cup and mixed with the acetylcholine solution after equilibrating at 37°; in the experiments with red blood corpuscles, the centrifuged corpuscles were taken up in a blood pipette and treated in exactly the same way as the serum. The rate of liberation of CO_2 was obtained by plotting the manometer readings against time for the first 45 min. and drawing a line through the points. Estimations were frequently done in duplicate and the two values generally agreed to within 3 % with acetylcholine as substrate and 10 % with the aliphatic esters. In the experiments with eserine 0·35 c.c. 1/10,000 eserine sulphate was added to 2·65 c.c. diluted serum and the mixture allowed to stand for 20 min. before adding the acetylcholine. The experiments with methyl butyrate and tributyrin were carried out in exactly the same manner except that 0·1 c.c. of the pure substrate was put in the side tube in place of the acetylcholine solution: the total volume was kept the same. The accuracy was greater when the activities were high than when low activities were obtained.

Esterase activities are expressed as micro-equivalents ($\mu E.$) of ester hydrolysed per c.c. of serum (or corpuscles) per hr. The activities were corrected for the spontaneous hydrolysis of acetylcholine which corresponded to 16·5 $\mu E./c.c./hr.$ under these conditions: the spontaneous hydrolysis of methyl butyrate and tributyrin was negligible. The choline esterase activities may be converted into the usual clinical units [Jones & Tod, 1935] by dividing by the factor 2·36.

RESULTS

Effect of muscular exercise on the serum choline esterase. Muscular exercise produced a significant rise in the serum choline esterase activity provided that the exercise was fairly vigorous and lasted not less than 5–10 min. The extent of the rise depended on the amount and nature of the exercise and differed in different subjects. Some typical figures are given in Tables 1 and 2 and in Fig. 1. The change was statistically significant when examined by Fisher's 't' test [1938]. In some experiments the serum choline esterase returned almost to normal again within 10 min., but in others it took considerably longer than this. That the rise in esterase activity was due to a choline esterase and not to some other esterase was shown by the complete inhibition by eserine (Table 2). The rise was shown in citrated and oxalated plasma as well as in serum.

Esterase changes in red blood corpuscles. The choline esterase in the red blood corpuscles is generally much more active than that in the serum. When the choline esterase activities were estimated in both serum and centrifuged blood corpuscles, before and after exercise, the rise in the serum was accompanied by

the activity of the blood corpuscles towards the aliphatic esters and also by (b) the appearance in the serum of an esterase which was not inhibited by eserine. The choline esterase and the ali-esterase did not pass at exactly the same rates from the corpuscles into the serum and the two enzymes appeared to shift independently of each other. Exercise increased the rate of hydrolysis by the serum of methyl butyrate and of tributyrin, but this was accounted for by the ali-esterase. When the ali-esterase was taken into account, it was clear that the
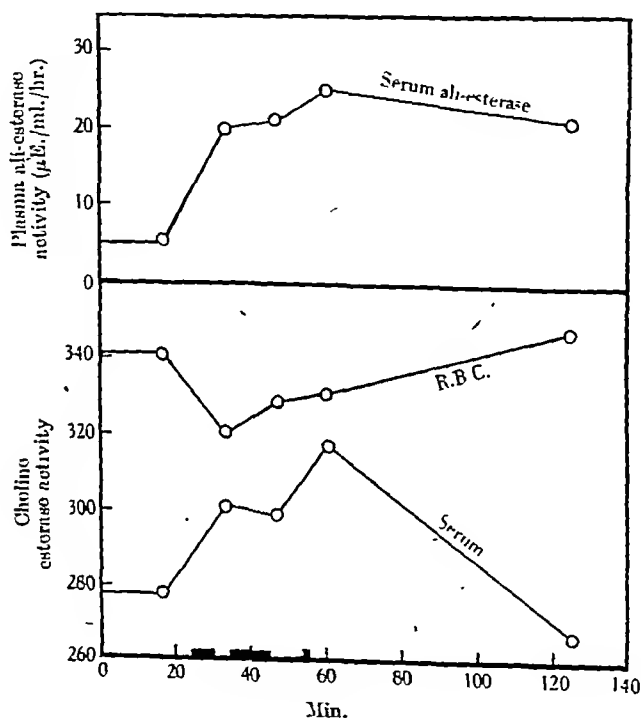
Fig. 1. Esterase changes in blood during exercise. Subject D. R. (72 kg.). Weights (4 kg.) lifted 100 times with right arm (520 kg.m.) at 25–30/min.; 100 steps climbed (6580 kg.m.) at 34–44/min.; ¼ mile run at 54–55 min. Esterase activities in μE./c.c. citrated plasma or packed red blood cells/hr. Methyl butyrate was the substrate for the ali-esterase. Capillary blood.

additional choline esterase appearing in the serum had no significant activity towards the aliphatic esters. This gave evidence that the esterase appearing in the serum during muscular exercise was not of the normal serum type, but was of the specific type, similar to that which occurs in the red blood corpuscles.

Failure to obtain a significant esterase shift after exercise of 5 or more min. duration was observed in only one out of thirty experiments. On this occasion the subject (D.R.) showed a high initial serum choline esterase activity of 321 which was not further increased by vigorous exercise: the ali-esterase activity

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TABLE 2. Esterase changes in plasma and corpuscles due to exercise on bicycle ergometer

All blood specimens were venous. Subjects P. 3, P. 5 and P. 6 were effort syndrome patients. The subjects were all adult males 60–80 kg. in weight. The figures represent activities (μE./c.c./hr.) with acetylcholine, methyl butyrate and tributyrin as substrates. The figures in brackets represent the activities obtained after the addition of 10^{-5} eserine sulphate. The bicycle ergometer was adjusted so that the work done was approximately 970 kg.m./min. during the first 10 min.; with subjects who were able to continue longer than this the work was then increased to approximately 1400 kg.m./min.

Subject	Dura- tion min.		Plasma μE./c.c./hr.			Blood corpuscles μE./c.c./hr.		
			Ac.ch.	Me. but.	Tribut.	Ac.ch.	Me. but.	Tri- but.
P. 3	3	(a) Resting	72 (0)	—	—	204 (0)	—	—
		(b) After ex.	91 (0)	—	—	281 (0)	—	—
		(c) 10 min. after ex.	95 (0)	—	—	262 (0)	—	—
P. 5	7	(a) Resting	192	—	101 (0)	355	—	72
		(b) After ex.	204	—	113 (4)	334	—	65
Dr R.	12	(a) Resting	55	25	36	355	34	50
		(b) After ex.	81	32	46	337	35	54
		(c) 10 min. after ex.	74	25	40	334	38	61
Dr J.	13	(a) Resting	65	31	64	365	42	68
		(b) After ex.	107	50	80	320	31	54
		(c) 13 min. after ex.	95	47	71	306	38	61
L.C.	8	(a) Resting	62	34 (1)	—	376	41	—
		(b) After ex.	80	41 (8)	—	338	38	—
		(c) 10 min. after ex.	71	36 (12)	—	340	33	—
H.B.	12	(a) Resting	140	57 (0)	—	329	53	—
		(b) After ex.	156	59 (4)	—	315	53	—
		(c) 10 min. after ex.	145	67 (9)	—	301	34	—
P. 6	8	(a) Resting	150 (0)	61 (0)	—	387	42	—
		(b) After ex.	176 (0)	78 (12)	—	322	35	—
		(c) 5 min. after ex.	160 (0)	66 (5)	—	339	40	—

acts preferentially on simple aliphatic esters and glycerides, such as methyl butyrate and tributyrin [Richter & Croft, 1942 b].

The serum and corpuscle choline esterase differ considerably in their specificity in that only the former is active towards the aliphatic esters. It appeared that it should be possible to obtain further evidence as to the source of the additional choline esterase appearing in the serum during exercise by testing with methyl butyrate and tributyrin whether this additional enzyme is of the specific or of the unspecific variety. Any conclusions as to the specificity must be based on the comparison of relatively small differences between two or more estimations, and it was clear that experiments of this kind would be difficult since they must make very considerable demands on the accuracy of the experimental technique: all estimations were therefore carried out in duplicate or triplicate. The figures obtained with methyl butyrate and tributyrin are given in Table 2.

In analysing the figures in Table 2 it should be noted that some of the aliesterase as well as the choline esterase passed from the red blood corpuscles into the serum during muscular exercise. This was shown by (a) the decrease in

the bodily reaction (neuro-muscular) to the lowered temperature. Since the esterase shift was observed to occur normally in the same subject at other times, the non-appearance of the shift under these conditions did not appear to conflict seriously with the main conclusions.

Corpuscular fluid equilibrium and esterase shift. Some individuals show a transient increase in the mean corpuscular diameter immediately after exercise [Wiechmann & Schürmeyer, 1925; Price-Jones, 1933]. This effect has been attributed to the passage of fluid from the plasma into the corpuscles owing to the increased CO_2 tension of the blood. Although the effect is small and apparently occurs only at exhaustion, it might account for an apparent lowering of the corpuscular esterase content that would be significant in the exercise experiments that have been described.

The passage of fluid from the plasma into the corpuscles would be shown (a) by a lowering of the haemoglobin concentration per unit volume of corpuscles, (b) by a change in the haematocrit, and possibly (c) by an increase in the total plasma protein concentration, if equilibrium with the tissue fluids was not attained. Experiments in which these three factors were measured (Table 3) confirmed the esterase shift but failed to detect any significant change in the mean corpuscular volume. It was further observed that whereas the effect observed by Wiechmann & Schürmeyer and Price-Jones was very transient and disappeared in 10 min. after the cessation of exercise, the loss of esterase from the red blood corpuscles always persisted for 10 or more min. (Table 2). It was concluded that changes in the fluid equilibrium could not account for the esterase shift that was observed in these experiments.

The effect of factors other than exercise on the choline esterase activity

The transfer of choline esterase from corpuscles to serum as a result of muscular exercise might be secondary to some biochemical change occurring in the blood at the same time. The following experiments on the subject D.R. were done to test this possibility, but no significant change in the serum choline esterase was observed.

Adrenaline (1 mg.) was injected and $3\frac{1}{2}$ hr. later a second dose (0.5 mg.) was given. *Ergotamine* (1 mg. 'Femergin') was taken by mouth. *Histamine* (1 mg. of acid phosphate) was injected subcutaneously. The *acid-base balance* of the blood was changed by overbreathing (37·4 l./min. for 10 min.), by underbreathing (3·8 l./min. for 10 min.) and by rebreathing from 5 l. of a mixture of 5 % CO_2 in oxygen for 7 min. In the last experiment the pulse rate had risen to 98 and sweating, nausea, giddiness and tremor supervened.

Anoxaemia. Local anoxaemia produced by passing a ligature round the arm so as to effect a temporary stoppage of the circulation, caused a sharp rise in the serum choline esterase. This was also observed by McArdle [1940]. The rise was of the same order of magnitude as that occurring in muscular exercise, but

was 24. This experiment was done on a particularly cold day before the central heating of the laboratory had been turned on. It appeared likely that the failure of the serum choline esterase to rise with exercise was due to its being already abnormally high before the beginning of the experiment as a result of

TABLE 3. Exercise experiments giving esterase changes with plasma or serum protein and corpuscle haemoglobin concentrations

Haemoglobin estimations were done by the Haldane method: protein by the biuret method. Exercise indicated by asterisk in Exp. 3 consisted of running $\frac{1}{4}$ mile in about 1 min.

(1) Subject D.R.

Time min.	Work kg.m.	Plasma C.E. μE./o.c./hr.	Plasma proteins mg./100 c.c.	Corpuscles		Whole blood	
				C.E. μE./c.c./hr.	Hb. mg./c.c. cells	C.E. μE./c.c./hr.	Haemato-crit % of r.b.c.
0	—	263	7·9	347	337	141	48
1–10	7820	—	—	—	—	—	—
13	—	288	8·0	329	334	139	50
16–34	Resting	—	—	—	—	—	—
34	—	257	8·0	346	334	139	49

(2) Subject P.G.C.

Time min.	Work kg.m.	Serum C.E. μE./o.c./hr.	Serum proteins mg./100 c.c.	Corpuscles		Whole blood	
				C.E. μE./c.c./hr.	Hb. mg./c.c. cells	C.E. μE./c.c./hr.	Hb. mg./c.c. blood
0	—	121	7·0	291	262	217	134
7–15	8200	—	—	—	—	—	—
17	—	135	7·0	275	262	217	141
20–75	Resting	—	—	—	—	—	—
75	—	116	7·0	301	262	217	138

(3) Subject D.R.

Time min.	Work kg.m.	Serum			Corpuscles	
		C.E. μE./c.c./hr.	Proteins mg./100 c.c.	Ali-esterase μE./c.c./hr.	C.E. μE./c.c./hr.	Hb mg./c.c. cells
0	—	278	7·8	5	341	345
8–13	520	—	—	—	—	—
16	—	301	8·0	20	320	350
18–28	6580	—	—	—	—	350
30	—	299	8·0	21	329	—
37–38	*	—	—	—	—	345
43	—	318	8·3	25	331	—
45–108	Resting	—	—	—	—	340
108	—	268	8·0	21	348	—

(4) Subject patient 9

Time min.	Work kg.m.	Plasma			Corpuscles	
		C.E. μE./c.c./hr.	Proteins mg./100 c.c.	Ali-esterase μE./c.c./hr.	C.E. μE./c.c./hr.	Hb mg./c.c. cells
0	—	162	6·2	—	393	326
0–5	4850	—	—	—	—	328
5	—	174	6·0	—	376	—
5–13	Resting	—	—	—	—	328
13	—	155	6·0	—	385	—

Site of the choline esterase shift during exercise

It was not necessary to exercise the whole body in order to produce a large choline esterase shift: a maximal shift could be produced even when the exercise was limited to one arm (Table 1). This made it unlikely that the observed change in the blood choline esterase occurred locally in the exercised muscle, since only a small-fraction of the systemic blood could flow at any time through an exercised arm: the local change in the blood traversing the arm would have to be very great to account for the considerable change observed in the whole systemic blood. A further experiment to elucidate this point was carried out by exercising the muscles of one arm and comparing the choline esterase activity of the blood taken from the exercised arm with that of blood from the other arm. The superficial circulation through the skin was reduced to a minimum by immersing the hand in cold water. Blood was taken from the median cubital vein while exercising the flexor and extensor muscles of the left forearm and hand by alternately clenching the fist and extending the fingers with sufficient vigour to produce rapid fatigue:

	Serum c.e. μE./c.c./hr.	Serum proteins mg./100 c.c.	Corpuscle c.e. μE./c.c./hr.
Left arm	282	8·0	318
Right arm	270	7·5	318

(Subject: D.R. Muscles of left forearm and hand exercised for 2 min. before taking blood from the median cubital veins.)

No significant difference was found in the choline esterase activities of the serum or corpuscles of blood taken from the two arms. The very slightly increased activity of the serum from the exercised arm may be attributed to the slight local concentration of the serum proteins due to oedema in the muscle which is known to occur during vigorous exercise.

It is concluded that the shift in the blood choline esterases during muscular exercise does not occur locally in the muscle exercised; it may occur in some visceral organ, or it may occur uniformly throughout the whole of the circulating blood as a result of the chemical changes in the blood due to exercise.

Effect of chemical factors on the blood choline esterase distribution in vitro

In view of the chemical changes in the lactate, potassium and other metabolites during exercise, the effect of a series of substances on the choline esterase distribution between the corpuscles and plasma was tested in vitro. The chemical substances were incubated at 37° with fresh oxalated whole blood and the choline esterase activities of the plasma and corpuscles were estimated (Table 6).

When sufficient neutral salt (NaCl, KCl or Na_2SO_4) was added to make the plasma strongly hypertonic there was an apparent change in the choline

the effect differed from that occurring during exercise in that the esterase activity of the red blood corpuscles showed no change (Table 4). Tests with aliphatic esters showed that there was also no shift in the ali-esterase, and the additional choline esterase found in the serum was of the unspecific type. The

TABLE 4. Effect of stasis on serum and corpuscle esterase activities

Venous stasis for 10 min. was obtained with a sphygmomanometer cuff round the arm inflated to 100–120 mm. pressure. Venous blood was taken in Exp. 3 and capillary blood (after clamping the brachial artery) in Exps. 1 and 2. The figures represent activities (μE./c.c./hr.) with acetyl-choline, methyl butyrate and tributyrin as substrates. The figures in brackets represent the activities obtained after the addition of 10^{-5} eserine.

				Serum μE./c.c./hr.			Blood corpuscles μE./c.c./hr.		
No.	Subject			Ao.ch.	Me.but.	Tribut.	Ao.ch.	Me.but.	Tribut.
1	P.G.C.	(a)	Resting	117	—	—	278	—	—
		(b)	After stasis	141	—	—	284	—	—
2	D.R.	(a)	Resting	282	—	—	346	—	—
		(b)	After stasis	396	—	—	352	—	—
3	D.R.	(a)	Resting	252 (0)	94 (2)	153 (0)	370	45	68
		(b)	After stasis	342 (0)	130 (4)	208 (0)	370	50	68

changes occurring during stasis were clearly different from those during exercise. It was noted that the ratio of blood corpuscles to serum was far greater than normal in the specimens collected during stasis. This suggested that the rise in serum choline esterase during stasis might be explained by the increased passage of fluid through the capillary walls.

Estimations of the serum proteins confirmed an increased concentration as a result of circulatory stasis, sufficient in amount to account for the observed rise in choline esterase activity. The figures (Table 5) contrasted with similar

TABLE 5. Comparison of the effects of exercise and of venous stasis on the serum choline esterase and total serum proteins

Total serum proteins estimated by the biuret method [Harrison, 1937]. Capillary blood in the stasis experiments: venous blood in the exercise experiments. In Exps. 3 and 4 oxalated plasma was used instead of serum.

No.	Subject			Choline esterase μE./c.c./hr.	Increase %	Total serum proteins mg./100 c.c.	Increase %
1	P.G.C.	(a)	Resting	117	0	7·5	0
		(b)	After stasis	141	20	8·7	22
2	D.R.	(a)	Resting	282	0	7·7	0
		(b)	After stasis	396	40	10·0	30
3	P. 8	(a)	Resting	168	0	7·1	0
		(b)	After exercise	183	9	7·2	1
4	P. 6	(a)	Resting	150	0	6·4	0
		(b)	After exercise	176	17	6·4	0

estimations made on sera taken before and after exercise, in which there was a rise in choline esterase activity without any corresponding increase in the total serum protein concentration.

cell membrane might free the enzyme from the surface and produce an artificial esterase shift. It was found in preliminary experiments that haemolysis of human erythrocytes suspended in serum became evident in about 3 hr. in the presence of 1/10,000 lysolecithin at 37° C. Incubation of the corpuscles under these conditions for 1½ hr., which should be sufficient to damage the cell membranes without actually haemolysing them, produced no significant esterase shift.

	Serum		Corpuscles	
	Incubated alone	With lysolecithin	Incubated alone	With lysolecithin
(1)	182	173	303	302
(2)	176	183	261	266

It appeared likely that the esterase shift was produced in vivo by the mediation of some chemical substance; but normal human erythrocytes gave no change in esterase activity when incubated in vitro with serum taken from a subject immediately after exercise.

Serum (from exercised subject)		Corpuscles	
Incubated alone	With corpuscles	Incubated alone	With serum from exercised subject
297	297	315	329

Incubation of the cells alone with serum produced no esterase shift in periods up to 30 min. There was also no return to normal in the esterase activities of sera and corpuscles collected after exercise on incubation for 2½ hr.

DISCUSSION

Evidence has been obtained that during muscular exercise there is a shift in the choline esterase from the red blood corpuscles to the serum. This occurs in man as a normal physiological process. The shift was observed to occur in a variety of different types of exercise which were not necessarily very strenuous. The time taken for a significant change to occur was as little as 3 min. The decrease in the choline esterase content of the red blood corpuscles amounted in some experiments to 16 or 17%. The percentage rise in the serum choline esterase depended on the initial concentration and the haematocrit; but when the initial serum choline esterase activity was low the rise amounted in some experiments to an increase of 50 or 60%. In addition to the choline esterase shift there also occurred a similar shift in the eserine-resistant ali-esterase, an enzyme which acts preferentially on the simple aliphatic esters and glycerides.

Vahlquist [1935] and Hall & Lucas [1937] reported that muscular exercise is without effect on the serum choline esterase. Croxatto, Huidobro, Croxatto & Salvestrini [1939] reported a rise in the blood choline esterase in cats under Dial anaesthesia on stimulating the motor nerves to a leg, but Trowbridge [1941] failed to verify this.

The apparent discrepancy between our results and those of Vahlquist, who

esterase activities of the plasma and corpuscles due to shrinkage of the cells and corresponding dilution of the plasma, but no evidence was obtained of any shift in the choline esterase distribution with any of the chemical factors tested.

TABLE 6. Effect of chemical factors on choline esterases in oxalated whole blood

Oxalated whole blood was incubated at 37° for 20 min. with and without the additions. With glutathione the incubation was for 40 min. During incubation with Na_2SO_4 the corpuscle volume decreased from 43 to 32 %. With CO_2 the gas was bubbled through for 10 min. The cholesterol was added in the form of a fine emulsion prepared by shaking a 20 % solution of cholesterol with a 20 % aqueous solution of lecithin and dialysing for 2 hr. against distilled water to remove the alcohol.

Addition	Conc. mg./c.c.	Plasma μE./c.c./hr.		Corpuscles μE./c.c./hr.	
		Alone	With addition	Alone	With addition
NaH_2PO_4	5	100	102	256	249
Na_2SO_4	20	137	116	315	377
NaCl	15	100	71	256	313
KCl	20	100	81	256	306
CO_2	Sat.	196	192	303	314
Choline Cl	5	137	142	315	314
Na lactate	2	137	140	315	308
Na ascorbate	5	137	140	315	310
Glutathione	10	119	121	270	264
Lecithin	5	176	182	261	261
Lecithin and cholesterol	5	176	182	261	258

It was noted by previous investigators [Alles & Hawes, 1940] and confirmed in this laboratory that when the choline esterase activity of the red blood corpuscles was measured, the same result was obtained whether the cells were previously haemolysed or not. This suggested that the corpuscle esterase might be mainly adsorbed or combined at the surface of the cells.

It appeared possible that the choline esterase might still be mainly associated with the cell membranes after haemolysis, but experiments to test this by haemolysing with distilled water or with ether and carefully centrifuging off the empty cell membranes showed that the whole of the enzymic activity passed with the haemoglobin into the supernatant fluid and no choline esterase activity was shown by the cell membranes.

	Choline esterase (μE./c.c./hr.)		
	Whole cells	Skins	Supernatant solution
Haemolysis by distilled water	340	0	301
Haemolysis by ether	233	0	214

The absence of enzymic activity in the cell membranes did not disprove the view that the enzyme is normally adsorbed in part on the surface, since the membranes are manifestly damaged in the process of haemolysis.

Lysolecithin. If the choline esterase is mainly adsorbed on the cell surface, it appeared that an agent such as lysolecithin which progressively damages the

It is hard to believe that a protein such as the corpuscle choline esterase is able to pass with any degree of ease through the cell walls; it therefore appeared probable that the enzyme is mainly adsorbed or combined at the cell surface, from which it is partially released during exercise. However, the empty cell membranes which were obtained after haemolysis showed no choline esterase activity.

The apparent changes in choline esterase activity of the serum and corpuscles during muscular exercise might be attributed to a diffusible coenzyme or to an inhibitor passing in the reverse direction from the serum into the cells. While these must be considered as formal possibilities, there is no evidence of the existence of any such inhibitor or coenzyme and it is unlikely that a factor of this kind would affect the eserine-resistant ali-esterase in precisely the same way. A shift in the choline esterase is therefore the simpler interpretation of the results.

The changes in the serum and corpuscle choline esterase during exercise are too large to be accounted for as a haemoconcentration effect, and similarly they would appear to be too large to be related to the sudden discharge during exercise of 'non-circulating' blood from the spleen. Adrenaline, which also causes contraction of the spleen, was without effect.

Assuming that our interpretation of the results is correct and that there is a shift of choline esterase and ali-esterase from the corpuscles to the serum, it would appear probable that this change is effected by some chemical metabolite released during exercise which elutes the enzymes adsorbed or combined at the surface of the corpuscles so that they pass into the plasma.

Physiological significance of serum choline esterase. Previous investigators have assigned to the serum choline esterase an important function in destroying acetylcholine entering the blood from the tissues and in so controlling the state of autonomic balance [Stedman & Russell, 1937; Antopol, Tuchman & Schifrin, 1937; Tod & Jones, 1937]. Schütz has attempted to relate the serum choline esterase activity to the state of activity of the central nervous system [1941].

Against these views it should be pointed out that the choline esterase activity of the serum is usually much lower than that of the blood corpuscles or of the tissues, where most of the destruction of choline esters may be presumed to occur. It is therefore unlikely that the changes that have been observed in the serum choline esterase activity can be very significant in affecting the acetylcholine content of the blood.

A number of attempts have been made by previous investigators to make use of the serum choline esterase activity as a clinical test and this work has added to our knowledge of its significance.

High serum choline esterase activities have been observed (a) in acute emotional states, (b) in thyrotoxicosis [Antopol *et al.* 1937], and (c) in 'post-concussional' states [Richter & Lee, 1942 b], while low activities have been

examined the serum of a man after 'an hour's hard tennis-playing', may be due to too long an interval being left between the exercise and the taking of the blood sample; the serum choline esterase may return to normal within 10-15 min. after exercise and the peak in the rise might easily have been missed. This might apply also to the work of Hall & Lucas. The experiments of Croxatto *et al.* are difficult to interpret since whole blood was used without stating the haematocrit; the choline esterase concentration is different in the serum and corpuscles and it is not clear which was concerned in the change observed.

Source of additional choline esterase. The additional choline esterase found in our experiments in the serum after exercise was shown to come from the red blood corpuscles, since the corpuscles gave a simultaneous fall in choline esterase which was similar in amount to the increase in the serum.

Further evidence of the source of the additional choline esterase in the serum after exercise was obtained by making use of the fact that the corpuscle choline esterase differs considerably from the serum choline esterase in its specificity towards different substrates [Richter & Croft, 1942 b]. Experiments with methyl butyrate and tributyrin gave figures which agreed with expectations in showing that the additional choline esterase in the serum after exercise resembles the corpuscle choline esterase rather than the normal serum choline esterase in its specificity.

Mechanism of choline esterase shift. It appeared possible that the choline esterase shift during exercise might be due to the change in acid-base equilibrium between the serum and corpuscles; but alteration of the acid-base equilibrium by vigorous overbreathing, underbreathing or by rebreathing 5 % CO_2 in oxygen produced no significant change in the choline esterase. Experiments with adrenaline and histamine showed again that the shift cannot be attributed to the liberation of these substances in the blood during exercise.

Anoxaemia produced by passing a ligature round the arm so as to effect a temporary circulatory stasis caused a rise in the serum choline esterase of the blood taken from the ligatured arm, but this effect was due to the local rise in the total serum proteins through oedema. It was different from the shift occurring during exercise, since there was no corresponding fall in the corpuscle choline esterase activity and no shift in the ali-esterase.

It is unlikely that the choline esterase shift during exercise occurs locally in the active muscles since the blood taken from vigorously acting muscles showed no significant difference in choline esterase from that taken from muscles that were inactive.

Attempts to reproduce the choline esterase shift in vitro by adding chemical substances to oxalated whole blood at 37° gave negative results with lactic acid, sodium acid phosphate and a series of other metabolites which change in concentration during muscular exercise. These experiments gave no clue as to the mechanism of the choline esterase shift.

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observed (d) in catatonic stupor [Tod & Jones, 1937], (e) in the extreme debility associated with advanced tuberculosis, carcinoma, anaemia and liver disease' [McArdle, 1940], and (f) after administering narcotics [Ahlmark & Kornerup, 1939; Schütz, 1941]. It is evident that the conditions associated respectively with high and with low serum choline esterase activities in man are contrasted with respect to the prevailing conditions of autonomic and of neuro-muscular activity. A case might be made out for associating the serum choline esterase, activity with either of these two factors. In muscular exercise both autonomic and neuro-muscular activity again occur together and are associated with a serum choline esterase rise.

It is clear that further data must be obtained before the significance of the serum choline esterase activity can be established; but the results of the present investigation show that muscular activity is one of the factors that must be taken into account in clinical work on the serum choline esterase activity.

SUMMARY

1. The serum choline esterase activity is raised during muscular exercise in man.

2. The choline esterase of the blood corpuscles falls during muscular exercise and this may account for the rise in the serum choline esterase.

3. Some of the ali-esterase in the red blood corpuscles also passes into the serum during muscular exercise.

4. The serum choline esterase activity is not raised by the administration of adrenaline, ergotamine or histamine or by overbreathing, underbreathing or rebreathing the expired air.

5. The serum choline esterase activity is raised during circulatory stasis.

6. The rise in serum choline esterase during circulatory stasis is due to the increase in the concentration of the serum proteins: it is not related to the rise during muscular exercise.

7. The mechanism of the choline esterase shift and the significance of the serum choline esterase activity are discussed.

o that two strips of intestine could be observed simultaneously under identical conditions. The aspirator bottles were so placed that the hydrostatic pressure in the lumen of the gut was about 2 cm. water.

The intestine was suspended at temperatures between 35 and 37° C. in glucose-free Tyrode saturated with oxygen. As shown by Feldberg & Solandt [1942], the reactions of the two muscular coats are affected very differently by the immersion in a glucose-free medium: whereas the spontaneous activity of both is much reduced or abolished, response to drugs is strongly inhibited in the longitudinal but hardly diminished, and occasionally even enhanced, in the circular muscle. The condition of 'exhaustion' through lack of carbohydrate seemed, accordingly, particularly suited for an investigation of the responses of the circular coat.

Results

Potassium chloride

Powerful contraction of the circular coat is obtained, as described by Feldberg & Solandt [1942], by the addition to the glucose-free Tyrode of small quantities of muscarine. Very similar effects were seen in the present investigation with KCl.

Smaller doses (25–60 mg. in 75 ml. bath) usually produce a rhythmical outburst of activity (Fig. 1), whereas larger quantities (75–100 mg.) may cause a prolonged tonic contraction on which very small rhythmical movements are superimposed (Fig. 2). The figures also show how weak, in comparison with the circular muscle's responses, are the reactions to KCl of the longitudinal coat after exhaustion through lack of glucose. Whereas 20µg. of atropine are sufficient to prevent the action of muscarine on the circular coat, 20, 40 and 100µg. do not abolish, though they may slightly reduce, the contractions elicited by KCl. Fig. 1 illustrates the effect of two consecutive doses of 60 mg. KCl: the first one is on the untreated preparation, the second 5 min. after 40µg. atropine. The effect on the circular coat is unchanged, that on the longitudinal coat even enhanced. A further dose of KCl was given 84 min. later, following 100µg. atropine, and there was still a powerful response of the circular muscle.

Nicotine was found to be equally incapable of abolishing the effect of KCl, although doses were employed which paralysed the circular muscle to further additions of nicotine. Successive additions of four doses of 2 mg. nicotine acid tartrate, for instance, had rendered the strip entirely insensitive to the third and fourth dose of nicotine, whereas a large response followed the addition to the bath of 40 mg. KCl.

The persistence of the potassium chloride effects in the presence of paralysing doses of nicotine strongly suggests that their action is on the muscle itself. The quantities of KCl necessary to elicit a response are not sufficiently large

THE SITE OF ACTION OF SOME DRUGS CAUSING STIMULATION OF THE CIRCULAR COAT OF THE RABBIT'S INTESTINE

By MARTHE VOGT, *From the Pharmacological Laboratory of the College of the Pharmaceutical Society*

Observations on suprarenalectomized rabbits had shown that abnormal responses to certain drugs were sometimes exhibited by the circular coat of the small intestine. For a correct analysis of the results, however, too little was known of the circular muscle's normal behaviour. Experiments on the pharmacology of the circular coat were, therefore, carried out in the normal rabbit, and the results are presented in this paper.

METHODS

The rabbits were killed by a blow on the neck, the jejunum excised and immersed immediately in glucose-free Tyrode's solution. Two lengths of gut measuring 5–6 cm. were suspended in a double organ bath for observation, and the remaining piece of intestine kept at room temperature for periods up to 5 hr. to supply more strips if required. It was found that the results on different strips were only insignificantly changed by keeping the intestine at room temperature for a few hours, whereas they were strongly modified by cooling it down in the refrigerator for the same length of time. The gut was, therefore, only placed in the ice chest if the object of the experiment was observation of the changes produced by cold.

In all experiments, simultaneous record was made of the movements of the longitudinal and the circular coat by means of the method devised by Trendelenburg [1917]. In this technique, the activity of the circular coat is measured by changes in intestinal volume. One end of the strip of gut is tied over a piece of glass tubing connected to an aspirator bottle partly filled with Tyrode's solution and leading to a volume recorder. The other end is ligated and attached to the lever recording the activity of the longitudinal coat. The volume recorder registers movements of fluid in the bottle produced by circular muscle activity; it consisted of either a piston recorder or a small Krogh chamber. Details about the interpretation of the records can be found in the paper by Feldberg & Solandt [1942]. The organ tank contained 2 baths of equal volume (75 ml.); levers for the longitudinal muscle and volume recorders were also duplicated,

superimposed on a strong tonic contraction. In the interval between tracings *a* and *b*, 20μg. of atropine followed by 10μg. of eserine were given: the eserine had no effect. (This dose of eserine, in the absence of atropine, invariably caused stimulation of the circular coat." Examples of the action are given in Figs. 4 and 5 of the paper by Feldberg & Solandt [1942].) The eserine was
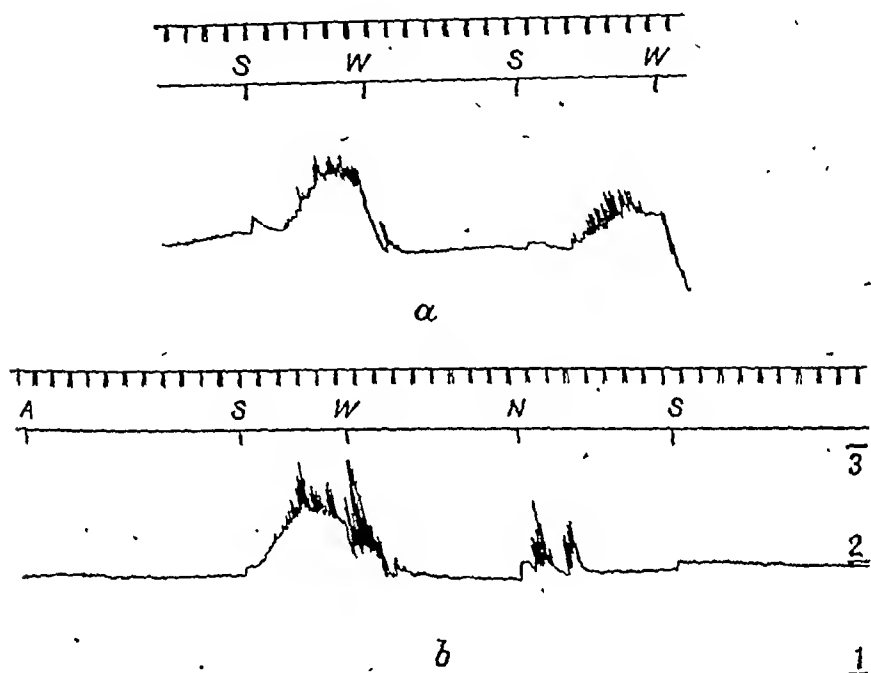
Fig. 3. 4. v. 42. Male rabbit, 1·7 kg. Strips of jejunum kept for 3½ hr. in Tyrode at room temperature, then suspended in warm, oxygenated, glucose-free Tyrode. Pendulum movements have nearly subsided 30 min. later, when a first dose of 160 mg. NaCl is given. Record starts 7 min. later. Intestinal volume recorded with Krogh chamber. Calibration in ml. Time: 30 sec. Volume of bath: 75 ml. Tracing *a*: *S*, 160 mg. NaCl; *W*, strip washed. Interval of 23 min., during which a first dose of 20μg. atropine sulphate followed by 10μg. eserine sulphate is given. The action of the eserine is prevented by the atropine. Tracing *b*: *A*, second dose of 20μg. atropine sulphate; *S*, 160 mg. NaCl; *N*, 2 mg. nicotine acid tartrate.

washed out and another dose of 20μg. atropine added at *A* (tracing *b*). This was followed by 160 mg. NaCl (*S*), and it is obvious that the effect was in no way diminished. At *N*, 2 mg. of nicotine were added to the bath. They caused an evanescent outburst of activity. Four minutes later a dose of NaCl was completely ineffective.

Inhibition of the sodium chloride stimulation was, however, also caused by larger amounts of atropine. Thus 40μg. atropine, for instance, nearly abolished the response in a strip of intestine which had reacted vigorously to the salt after treatment with 20μg. atropine.

to explain the effect as being caused merely by increased tonicity of the organ bath, since stimulation by hypertonicity could only be expected with doses
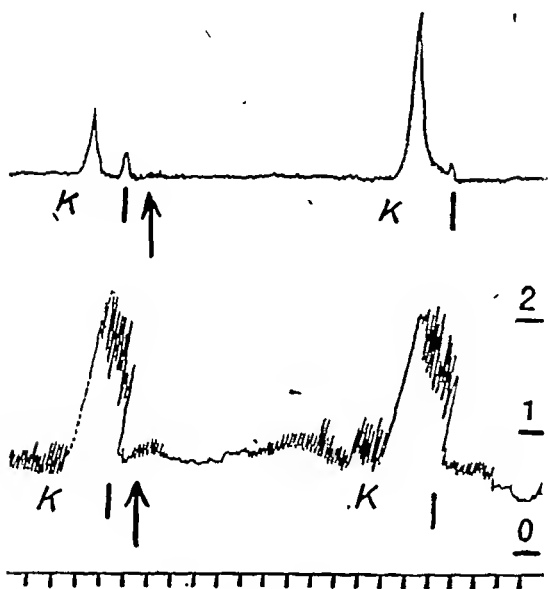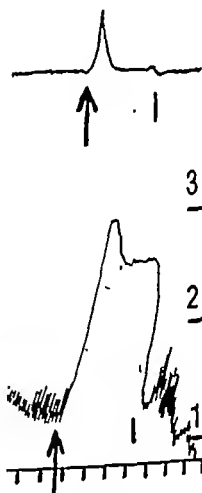
Fig. 1.   Fig. 2.

Fig. 1. Four-months-old rabbit, 0·93 kg. Piece of intestine (length 4 cm.) suspended in warm, oxygenated, glucose-free Tyrode. Record starts 35 min. later, when the pendulum movements have ceased. Upper tracing: movements of longitudinal muscle, amplification 4·5. Lower tracing: intestinal volume recorded with Krogh chamber. Scale in ml. Time: 30 sec. Size of bath: 75 ml. 60 mg. KCl are added to the bath at K and washed away at the strokes. At the arrows, 40μg. atropine sulphate are added to the bath.

Fig. 2. Same rabbit as that of Fig. 1. Piece of jejunum, 4 cm. long, suspended for 45 min. in warm, oxygenated, glucose-free Tyrode. Tracings and scale as in Fig. 1. 100 mg. KCl are added to the bath at the arrows, and washed away at the strokes.

at least five times the size of those used in the experiments just described. The effect is evidently analogous to the stimulation observed by Brown [1937] on normal or denervated striated muscle.

Sodium chloride

The addition of sodium chloride to the intestinal strip suspended in glucose-free Tyrode proved unexpectedly to have a potent stimulating effect on the circular coat. The quantities used were such as to increase the tonicity of the bath by about 20 %. An example is given in Fig. 3. (The movements of the longitudinal coat are not reproduced as they were negligible.) Tracing a represents the effect of two doses of 160 mg. salt (one dose had already been given before the record started). After a latent period of about a minute, during which there was a slight rise in tone, rhythmic activity developed

Sodium lactate

Feldberg & Solandt [1942] have shown that large doses of sodium lactate, though they are not utilized as carbohydrate, stimulate the circular coat. The effect is most obvious in the preparation depleted of its energy stores by prolonged activity in a glucose-free medium. The present experiments were carried out with a view to finding out which structures are affected by the lactate.

The amounts of lactate required in order to obtain regular effects are so large that they would increase the tonicity of the bath by 20 % if added to the ordinary Tyrode solution. As demonstrated in the last paragraph, an increase in tonicity of that magnitude will usually in itself stimulate the circular coat. The lactate was therefore given in a sodium chloride-free Tyrode, in which equimolecular amounts of lactate replaced the NaCl. In order to give a dose of 300 mg. lactate, for instance, 20 ml. of that Tyrode solution, in which the chlorine ion was substituted by the lactate ion, were warmed and added to the partly emptied bath. An example is represented in Fig. 5. The tracing is the record of the circular coat. At the first L, 300 mg. of lactate were added to the bath and caused a considerable rhythmic activity while the longitudinal muscle remained quiescent. The activity persisted for some time after the drug had been washed out (at W); this after-effect is frequently observed, and is more clearly seen in the figure after the second and third dose than after the first one, as this was very soon followed by a dose of 5 mg. nicotine acid tartrate (N_5). That this was not a paralysing dose is shown by the persistence of the activity in the circular muscle during the $7\frac{1}{2}$ min. following its application. The nicotine was then washed out, and a second dose of lactate added to the bath: the effect was somewhat larger than the previous one. Ten mg. of nicotine were then given, which succeeded in diminishing, though not in abolishing, the contractions of the circular muscle after an initial powerful stimulation. The nicotine was left in the bath and lactate was again given $6\frac{1}{2}$ min. later; a further increase of the effect resulted. The gut was then frequently washed and, 11 min. later, treated with another 10 mg. of nicotine. This time a short outburst of rhythmic contractions was followed by nearly complete paralysis of the circular coat. The nicotine was washed away and immediately followed by a fourth dose of sodium lactate. There was no response whatever; in a state of nicotine paralysis the strip had become insensitive to lactate. These results are reflected to a small extent in the longitudinal muscle: no response was obtained from the first two doses of lactate, but a slight activity was seen to result from the third dose which caused a very strong response in the circular coat. After the fourth application no response was seen in either coat. It may be mentioned that the doses of nicotine required to produce paralysis were unusually high in this preparation.

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Hence, stimulation by sodium chloride can be abolished by nicotine but is unaffected by atropine in doses which prevent the action of eserine or muscarine. This result was confirmed in other experiments. The sensitivity of different intestinal strips, however, to the action of salt is rather variable, as also is the latent period between addition of the drug and response. Furthermore, successive doses of NaCl do not always elicit similar responses, as in some preparation the effect gradually disappears.

The next question which arose was whether the stimulation by salt resulted from a specific effect of the sodium ion or whether it was merely a consequence of the hypertonicity of the solution surrounding the gut. The answer could easily be obtained by seeing whether solutions of inert substances of the same
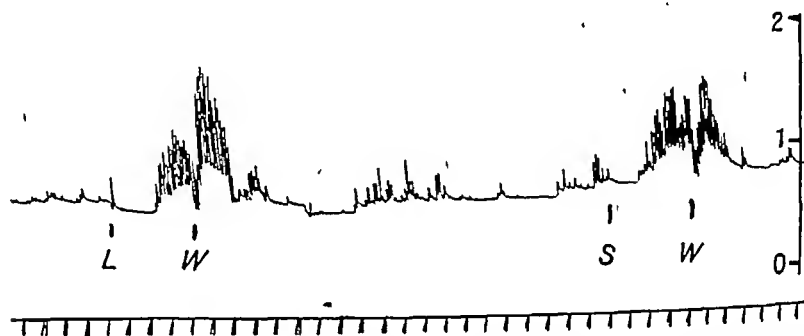
Fig. 4. 20. v. 42. Four-months-old rabbit. Piece of intestine kept for 2 hr. 50 min. in Tyrode at room temperature, then suspended in warm, oxygenated, glucose-free Tyrode. Record starts 35 min. later, when the pendulum movements have almost ceased. Record of circular coat with Krogh chamber. Scale in ml. Time: 30 sec. Volume of bath: 75 ml, L, 1·85 g. lactose; W, bath washed out; S, 1·85 g. sucrose.

tonicity would produce similar effects. From experiments by Rona & Neukirch [1912] and Feldberg & Solandt [1942], it was known that disaccharides have no effect on the longitudinal muscle. It was, therefore, likely that they could be used for our purpose.

The amount of disaccharide equivalent to 160 mg. NaCl is 1·85 g. A 50 % solution of lactose and a 90 % solution of sucrose were prepared and the required volumes added to the bath. Fig. 4 illustrates the result: after a latent period of about a minute, during which any activity of the circular coat previously present ceases, there is an outburst of rhythmic contractions very similar to that shown in Fig. 3 to follow the addition of NaCl. The longitudinal muscle (not reproduced here) was inhibited during the whole period of contact with the sugar solution. There is little doubt, therefore, that the stimulation observed when 160 mg. salt were added to the bath is an effect of hypertonicity. The probable site of this stimulation will be discussed later.

DISCUSSION

The responses to hypertonic solutions and to sodium lactate have several features in common: they are rhythmic contractions, frequently followed by a short period of activity after the drug has been washed away; they are insensitive to doses of atropine which abolish the effects of parasympathomimetic drugs: their actions are frequently enhanced by nicotine in moderate doses and are always prevented by amounts of nicotine which paralyse the gut to the addition of further nicotine. In this context it is interesting to remember the observations by Bayliss & Starling [1899] on the small intestine of the dog. After splanchnotomy, stimulation of the vagi caused increased intestinal activity, which was not abolished by doses up to 30 mg. atropine, but disappeared after injection of 3 mg. nicotine. The circular coat was much more powerfully stimulated than the longitudinal muscle. The resemblance between vagal stimulation (in its preferential activity on the circular coat and in its sensitivity to drugs) and the effects of hypertonic solutions or sodium lactate is evident: the most likely conclusion is that the site of action of salt and lactate is Auerbach's plexus. If the action of these substances is on the nervous elements, the failure of some preparations to respond repeatedly to the same stimulus is not surprising, as fatigue or paralysis are readily elicited in nervous structures.

There is an interesting quantitative difference in the sensitivity to atropine of the vagus stimulation in the anaesthetized dog on the one hand, and application of the drugs in question to isolated pieces of intestine on the other: vagus stimulation remains effective even after excessive doses of atropine (30 mg.), whereas addition to the bath of two or three times the amount of atropine necessary to abolish the action of parasympathomimetic drugs will decrease or inhibit the action of salt or lactate. The failure of atropine to prevent the effect of vagal stimulation has been explained by Dale & Gaddum [1930] on the assumption of a close proximity of the site where the acetylcholine is liberated to the place where it causes stimulation. Reaction between the atropine and the region of the muscle excited by acetylcholine is thus prevented. It would appear that in the isolated preparation suspended in an unphysiological medium (Tyrode), this protecting structure is damaged and atropine, provided it is given in sufficient quantities, will get access to and react with the acetylcholine sensitive 'receptors' in the tissue.

The cooled preparation

In the dying tissue, nervous structures are known to succumb more rapidly than the musculature. The fact that pendulum movements still occur in rabbit's intestine kept for several days in the refrigerator has been used by Gunn & Underhill [1914] as an argument for their muscular origin. It seemed

Atropine had the same influence on the stimulation by lactate as it had on the effects of hypertonic sodium chloride. Twenty μg. of atropine sulphate, which abolished the response to eserine, did not alter the effect of lactate, 60μg. atropine, however, diminished it. Care has to be taken in carrying out these
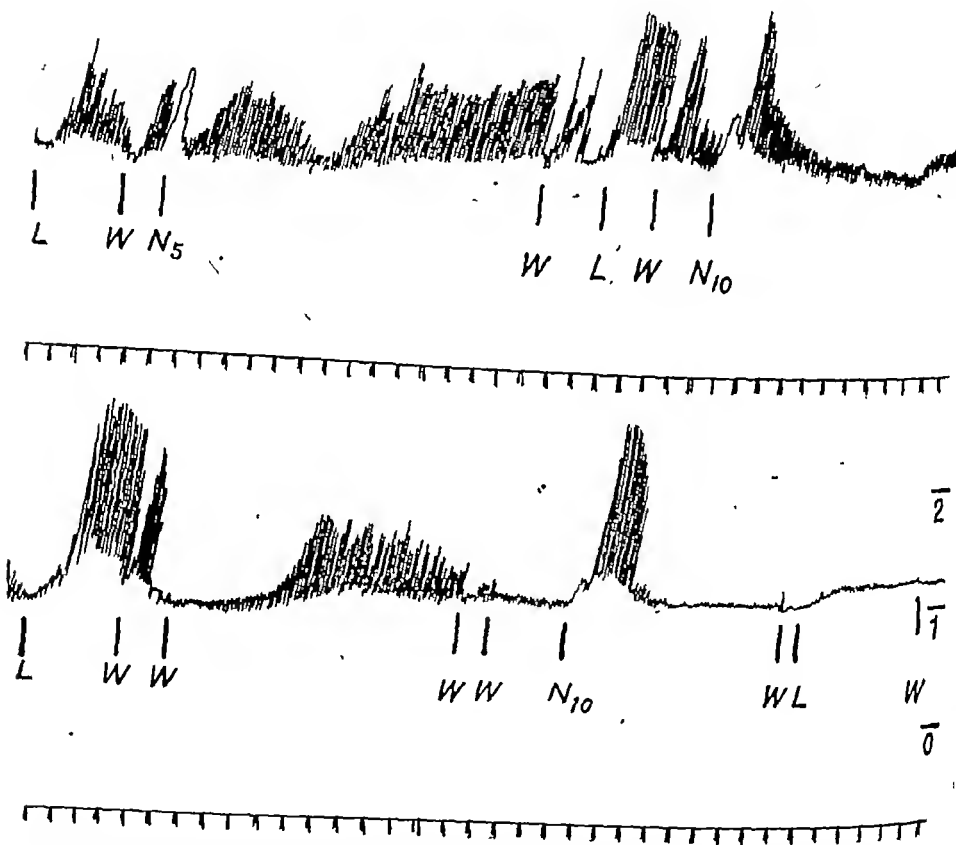
Fig. 5. 7. v. 42. Female rabbit, 2·0 kg. Strip of jejunum kept for 4 hr. in Tyrode at room temperature, then suspended in warm, oxygenated, glucose-free Tyrode. Tracing starts 40 min. later. Movements of circular coat registered with Krogh chamber. Calibration in mL. Time: 30 sec. Size of bath: 75 mL. L, 300 mg. sodium lactate in isotonic solution; W, strip washed; N_5, 5 mg., N_{10}, 10 mg. nicotine acid tartrate.

experiments to select strips of intestine which give a series of approximately equal responses to lactate. In some preparations the effect wears off rapidly and the influence of other drugs can, therefore, not be investigated. Moreover, the size of the initial response is very variable in different animals.

not affected by amounts of atropine which prevent the action of muscarine or eserine. Large doses of atropine, however, inhibit the effect. Similar responses of the circular coat are obtained, if hypertonicity of the bath is produced by the addition of lactose or of sucrose.

4. Sodium lactate, if applied to the intestine in such a way that it replaces part of the sodium chloride and therefore does not change the tonicity of the bath, stimulates the circular coat in a manner which resembles that of hypertonic solutions. Suitable doses are 300–450 mg. in 75 ml. The effect is abolished in the paralytic stage of nicotine poisoning but may be enhanced when paralysis has not yet been achieved. The effect is only abolished by atropine if the dose employed is several times that needed for the abolition of parasympathomimetic effects.

5. In the intestine undergoing slow disintegration at temperatures of 1° C., the response to a number of drugs was tested and seen to disappear in the following order:

Sodium lactate (300–450 mg.),

Sodium chloride (160 mg.) and eserine sulphate (10 μg.),

Nicotine acid tartrate (0·3–1·0 mg.),

Nicotine acid tartrate (2 mg.),

Muscarine (0·5–1 ml. solution 1:500) and potassium chloride (100 mg.).

6. The bearing of these results on the view that the mode of action of hypertonic solutions and of lactate is to stimulate Auerbach's plexus is discussed.

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.of interest, in order to try and confirm the view that the effects of hypertonic NaCl or of lactate are due to stimulation of nervous elements, to compare the rate of disappearance of these responses with that of drugs of known site of action in the preparation which was slowly dying in the refrigerator at about 1° C. The effects were compared of

300–450 mg. sodium lactate,	0·3–2 mg. nicotine acid tartrate,
160 mg. sodium chloride,	0·5–1 ml. muscarine[1] 1:500,
10 μg. eserine sulphate,	100 mg. potassium chloride.

The effect of lactate was the first to disappear; it was always absent if the intestine had been kept in the ice chest for 24–27 hr. Eserine and sodium chloride were the next to be abolished: they were usually present after 28, but always absent after 48 hr. At this latter period nicotine (0·3–1 mg.) had also become inactive, though a small effect on the tone of the muscle could still be elicited with 2 mg.; the rhythmic response had completely disappeared. Muscarine and potassium chloride had, at this stage, more effect on the gut than 2 mg. nicotine; the responses, however, were exclusively tonic in character.

The observation that the effects of lactate and hypertonic solutions are the first to be affected by cooling, supports the view that they are caused by stimulation of the nervous elements in the intestinal wall. The long persistence of the KCl effect, on the other hand, is in good agreement with the conclusion drawn at the beginning of this paper that its site of action is the muscle. Doses of nicotine which had an effect comparable in size to that of the other drugs employed, lost their efficacy a little later than NaCl or eserine, and earlier than muscarine or KCl. The persistence of a slight effect of 2 mg. nicotine after prolonged cooling is not at variance with the well-established fact that its site of action lies in the ganglion cells, as the dose of 2 mg. caused a much stronger reaction on the fresh preparation than any of the other substances used.

SUMMARY

1. The response to certain drugs of the circular coat of the rabbit's intestine was investigated in a preparation, in which the activity of the longitudinal coat had been much reduced by suspension in a glucose-free Tyrode solution.

2. 25–100 mg. KCl in a 75 ml. bath strongly stimulate the circular muscle. The responses are mainly rhythmic with smaller doses and become more tonic with larger ones. The effect is not abolished by atropine or by paralysing doses of nicotine. Its site of action is the muscle.

3. Sodium chloride, given in a quantity which increases the tonicity of the bath by 20 %, causes an outburst of rhythmic activity in the circular coat. The action frequently has a latent period of about a minute and is of variable size in different animals. It is abolished by paralysing doses of nicotine and

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[1] Natural muscarine, activity 1/450th of that of acetylcholine.

possible to show that this substance accumulates in the tissues of adrenalecto-mized animals. Experiments designed to test this possibility were recently carried out by Rose & Browne [1941] and by Wilson [1941]. The former workers reported that, while there was little change in the histamine content of blood, kidney or spleen, there was a moderate increase in liver and lung and a considerable increase in small intestine and stomach (see Table 3). Wilson reported an increase in blood histamine in adrenal-deficient rabbits.

The question of the relationship between adrenal cortical deficiency and tissue histamine appeared to require further investigation, and the work described here was undertaken in the hope that a more comprehensive study of the histamine content of the tissues of adrenalectomized rats in comparison with strict control experiments might throw more light upon the mechanism of cortin-histamine antagonism.

METHODS

Animals. Rats were used and were obtained chiefly from a small colony bred from the Wistar-strain albino colony of the Nutrition Laboratories of the College of the Pharmaceutical Society. In a few experiments rats kindly supplied by Dr S. K. Kon from the black and white strain colony of the National Institute for Research in Dairying were used. Young rats were mostly employed, wt. 30–70 g. (3–5 weeks old).

The rats were divided into litter-mate pairs; that is, each pair from the same litter, of the same sex and as nearly as possible of equal weight. Of each pair one animal was adrenalectomized and the other 'sham' operated, undergoing the same operative treatment except that the glands were not removed.

Operative technique. The rats were anaesthetized with nembutal and ether. The nembutal was diluted 10 times with physiological saline and injected intraperitoneally, 0·15–0·2 c.c. of the dilution to young rats, 0·5 c.c. to adult rats. Anaesthesia was completed by the administration of ether, immediately before operating, by placing the animal beneath a large funnel the stem of which was connected to an anaesthetic bottle containing ether. Air was blown through the ether by a syringe bulb. Using this method, excellent control of the degree of anaesthesia could be maintained.

Access to the adrenal glands was gained by a single, mid-dorsal longitudinal incision through the skin and two lateral oblique incisions through the muscle wall immediately above each kidney. The adrenal gland, together with surrounding connective tissue, was stripped away with forceps in a caudal direction. This technique was preferable to cutting away the gland, which increases the possibility of leaving cortical tissue behind. The muscle wall and skin were ligatured with silk. As soon as they could swallow, the rats were given 25 % glucose solution by mouth from a pipette, a treatment which has been found by Bruce & Wien [1940] to reduce the incidence of post-operative mortality.

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THE INFLUENCE OF ADRENAL CORTICAL DEFICIENCY ON THE HISTAMINE CONTENT OF RAT TISSUES

By P. B. MARSHALL (*Rammell Scholar*), *From the College of the Pharmaceutical Society, University of London*

During recent years evidence has accumulated of an antagonistic relationship between the cortical secretion of the adrenal gland and the histamine present in the tissues of the body. The beneficial results obtained in the clinical treatment with adrenal cortical extracts of shock conditions resulting from injury, burns and surgical operation appear to support this evidence, since these conditions are usually associated with a release of histamine [Barsoum & Gaddum, 1936]. Marmorston & Gottesman [1928] showed that the sensitivity of rats to histamine was increased after adrenalectomy. This observation confirmed the reports of other workers [Scott, 1927; Crivellari, 1927; Voegtlin & Dyer, 1925]. The discovery of the enzyme histaminase by Best & McHenry [1930] struck what at first seemed to be a fatal blow at the theory of cortin-histamine antagonism, and for a time the problem was not investigated further.

Subsequently it became evident that the comparatively slow histaminase reaction could not adequately account for the rapid disappearance of histamine in the body, particularly the large amounts released in certain pathological conditions. Interest was again aroused, therefore, in the possibility of an anti-histamine function of the adrenal cortex. Rose & Browne [1938] showed that the decreased resistance to histamine following adrenalectomy was associated with a loss of ability by the body to inactivate histamine. This report was followed by the discovery by Rose & Karady [1939] that adrenalectomized animals showed a decrease in the histaminase content of their lung tissue. Further, the same workers showed that both the ability to inactivate histamine and the normal histaminase content of the lungs were restored by the administration to the deficient animals of 'adreno-cortical substances' [Rose & Karady, 1939; Rose, Karady & Browne, 1940]. These results suggested that the anti-histamine activity of the adrenal cortex operates through the medium of histaminase.

If depriving animals of their adrenal cortex and its hormonal secretions reduces or abolishes their ability to inactivate histamine, then it might be

out in 150 c.c. Pyrex flasks heated on a sand-bath. The flasks had standard ground-glass necks. During the preliminary heating they were attached to Pyrex air condensers and afterwards transferred to a vacuum drying apparatus for removal of the acid. This apparatus accommodated four flasks, immersed in a water-bath, each flask being attached to a splash trap. Delivery tubes from the traps were brought together by T-joints and the vapour from the flasks led through a condenser-absorption chain consisting of two water-cooled condensers with ice-cooled receivers, in series, a calcium chloride absorption tower and a tube containing sodium hydroxide. The apparatus was exhausted by a power-driven vacuum pump. The absorption chain described was found to be satisfactory in preventing moisture, acid and alcohol from reaching the pump.

The rats were killed by a blow on the head except where brain was required for extraction; these were killed by an intraperitoneal injection of 5% sodium cyanide. The tissue required was rapidly removed, freed from superficial moisture and blood by gentle pressure between filter paper, and dropped into a weighed flask containing approximately the requisite volume of 10% HCl (150 c.c./20 g. tissue). Flask and tissue were then reweighed. More dense tissues such as liver, heart and kidney were finely chopped with a sharp knife before dropping into the flask. Other tissues were found to disintegrate quite readily in the whole condition, and it was considered desirable to avoid undue damage to the tissues. Blood was obtained by cardiac puncture and run immediately into HCl, without preliminary treatment with trichloroacetic acid. Sections of the alimentary tract were washed free from contents with physiological saline, except in determinations on the whole tract, when the contents were included. Whole animals were cut into small pieces immediately after killing, and extracted in 500 c.c. flasks. The extract was filtered and an aliquot part taken for evaporation.

The flasks were boiled gently for an hour. At the end of this period the tissues were completely disintegrated. The extract was dried in vacuo at an external bath temperature of 70–80° C. Some of the residual acid in the dry extract was removed by the addition of two separate portions of absolute alcohol, with subsequent distillation of the alcohol in vacuo at 50–60° C. The dry residue was extracted with three separate portions of cold distilled water and the extracts filtered through paper into clean, dry test-tubes. The tubes were plugged with cotton-wool and stored in a refrigerator at 1–2° C. until all the extracts from a group of rats had been prepared. The reaction of the stored extracts was sufficiently low (pH 1·5 or less) to prevent any bacterial growth during the period of storage, which never exceeded 5 days.

Estimation of histamine. The histamine in the extracts was measured by the isolated guinea-pig ileum method of Barsoum & Gaddum [1935]. Several modifications in the isolated-organ apparatus were devised or adopted during

Maintenance of operated rats. The operated and control rats were distributed singly into small cages each containing sawdust, a handful of wood shavings and a small pot of drinking water. All experimental rats were given the same diet from the time of weaning, consisting of a compressed cube preparation obtained from J. Thorley, Ltd., the composition and nutritional values of which have been described by Thomson [1936]. The cages were placed in a room maintained at about 70° F. For the first night after operation the animals were given physiological saline containing 0·5 % glucose to drink. This was replaced by tap water on the following morning. The animals were weighed daily.

Under these conditions young rats showed an increase in weight of 5–10 g. on the first morning after operation, due to the imbibition of large quantities of saline which is excreted more slowly than water. On the second morning (after 24 hr. on tap water) the adrenalectomized rats had usually returned to their original weight, while the controls were about the same weight as on the previous morning. From this time, the controls showed a steady normal increase in weight, while the adrenalectomized rats steadily lost weight. The latter developed symptoms of adrenal cortical deficiency usually between the 2nd and 4th day after operation. Any rats which survived this period generally increased steadily in weight and did not become deficient, probably due either to incomplete removal of the glands or to growth of accessory cortical tissue.

Symptoms of deficiency were preceded by a fall in body weight below the pre-operative level. This was followed by an increasing lassitude and a fall of temperature. Diarrhoea was not often observed in young rats. The final stage of the condition was complete prostration with occasional convulsions and irregular breathing, terminating in death. In young rats, under the conditions described, the complete course of the deficiency, from the first signs of lassitude to death, lasted only a few hours.

Adult rats showed greater fluctuations in weight among the controls, but in adrenalectomized adults there was a fairly regular decrease in weight once deficiency was established. The survival time of adult rats was longer than that of young rats, symptoms of deficiency never appearing in less than 3 days after operation.

Rats were taken for tissue extractions as they developed signs of deficiency. The limits of the conditions under which they were used ranged from loss of weight with slight lassitude to death with no rigor mortis. Animals which showed no signs of deficiency and those which had obviously been dead for some hours were rejected. Control rats were killed at the same time as the adrenalectomized litter-mates, and the two extractions carried out concurrently.

Extraction of histamine. The histamine was extracted from the tissues by the method of Best & McHenry [1930]. Hydrochloric acid extractions were carried

out in 150 c.c. Pyrex flasks heated on a sand-bath. The flasks had standard ground-glass necks. During the preliminary heating they were attached to Pyrex air condensers and afterwards transferred to a vacuum drying apparatus for removal of the acid. This apparatus accommodated four flasks, immersed in a water-bath, each flask being attached to a splash trap. Delivery tubes from the traps were brought together by T-joints and the vapour from the flasks led through a condenser-absorption chain consisting of two water-cooled condensers with ice-cooled receivers, in series, a calcium chloride absorption tower and a tube containing sodium hydroxide. The apparatus was exhausted by a power-driven vacuum pump. The absorption chain described was found to be satisfactory in preventing moisture, acid and alcohol from reaching the pump.

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Estimation of histamine. The histamine in the extracts was measured by the isolated guinea-pig ileum method of Barsoum & Gaddum [1935]. Several modifications in the isolated-organ apparatus were devised or adopted during

the course of the work. Tyrode's solution containing 1 mg./l. atropine sulphate was fed from a reservoir to a 2 c.c. bath via a 10 c.c. graduated pipette arranged so that the pipette could be filled from the reservoir and a measured volume of solution run into the bath from the pipette. A simple drip-feed device, as demonstrated by Huggett & Mant [1941], was introduced so that, during periods when the preparation was not in use, Tyrode's solution was fed drop by drop into the bath, the excess liquid escaping over the sides into the surrounding water. This prevented the spasm of the muscle which usually developed if the preparation were allowed to stand for long periods in unchanged solution.

Guinea-pigs, wt. 300–400 g. and of either sex, were used. The piece of ileum was taken from the lower end, close to the ileo-caecal valve, as this was found to be more sensitive to histamine than muscle from higher regions of the gut.

A solution of histamine acid phosphate equivalent to $10 \mu g$. of histamine base per c.c. was used as a standard. Small doses of this solution (0·001–0·01 c.c.) were added to the bath by means of a Wellcome 'Agla' all-glass syringe fitted with an 'Agla' micrometer attachment. Use of the syringe by hand was clumsy and, if much tilted to introduce the needle point into the bath, the piston slipped down the barrel and delivered more solution than was intended. It was therefore found more convenient to clamp the syringe in a horizontal position and connect the needle to it by a short length of fine rubber tubing, the needle being clamped with the open end held just below the surface of the solution in the bath. The point of the needle was ground flat to reduce the area of possible diffusion into the bath.

Immediately before estimation, each extract was adjusted to the reaction of Tyrode's solution with NaOH solution (5–20 % according to the acidity of the extract) and the final volume of the adjusted extract was recorded. The change of pH of the extract during the titration was followed by transferring minimal amounts of the liquid with a glass rod on to white filter paper and treating the stain with thymol-blue solution. This method was satisfactory even with dark-coloured extracts, since the dark stain remained at the point where the rod touched the paper, while a colourless ring of moisture spread outwards from the centre. The appearance of the first trace of green was taken as the end-point of the titration (about pH 8·0). The adjusted extract, or a suitable dilution of it, was added to the bath in doses of 0·02–0·2 c.c. by means of a 1 c.c. syringe graduated to 0·01 c.c.

In the earlier experiments a 5 min. interval was allowed between doses of standard or extract, but subsequently it was suggested [G. B. West, private communication] that the muscle would respond quite as regularly if the interval were reduced to 2 min. In view of the large number of extracts to be tested, the saving of time by this change was very important. The fluid in the bath was changed immediately after each dose of standard or extract and again before the administration of the next dose.

Under these conditions, most of the preparations would respond to a dose of histamine as low as $0.01 \mu g$. of base, though the usual working dose was $0.05-0.1\mu g$. The use of atropinized Tyrode's solution abolished the spontaneous activity of the muscle, so that a flat 'base-line' was recorded, which facilitated the comparison of contractions.

Control experiments. These were carried out on most of the tissues examined in order to determine whether, for each tissue, the addition of a known amount of histamine acid phosphate could be detected by the methods used. Tissues from normal rats were divided symmetrically and the halves extracted in separate flasks. To one flask was added, before extraction, the known amount of histamine in solution, the amount being roughly equal to the average proportional increase found in that tissue after adrenalectomy. The apparent histamine content per g. tissue was determined for both extracts, and the difference compared with the amount actually added to the second flask.

Water content determinations. Since adrenal cortical deficiency is accompanied by changes in the water content of some tissues, it was considered advisable to determine the relationship between the fresh and dry weights of samples of all the tissues used for histamine extraction. If the moisture content of a tissue altered significantly after adrenalectomy, the values for histamine content calculated in terms of fresh tissue alone would give an inaccurate picture of the true histamine change.

A portion of each tissue extracted was weighed on a tared watch-glass and dried to constant weight in an oven at $100°$ C. The histamine content of the tissues was calculated in terms of both fresh and dry material.

RESULTS AND CALCULATIONS

In the experiments described, 465 rats were adrenalectomized and an equal number of control animals were 'sham' operated. Of the 465 rats operated, 259 were actually used for tissue extractions; 111 were rejected because they had been dead too long when found, or for other reasons; 74 failed to develop adrenal cortical deficiency; and 21 died during or as a result of the operation.

Since there was considerable variation between individual determinations, the mean values for histamine changes between operated and control animals were determined by calculating the mean difference between the logarithms of the histamine contents of the tissues. This method of calculation was shown by Galton [1879] to give a more accurate mean value for a series of observations where the error is large, than would be obtained by the arithmetical method. The histamine content of all tissues (including blood) was expressed in μg. histamine base per g. fresh tissue.

The results are summarized in Table 1. All the tissues examined showed an increase of histamine with the exception of stomach and the whole gut. The value of the logarithmic method of calculation is particularly well illustrated

with liver. One of the nineteen determinations showed an increase in histamine of over 1000 % in the adrenalectomized animal. This abnormal value raises the arithmetic mean for the histamine change to about 900 %. Calculated by the logarithmic method, however, the value of the mean change is about 100 %, which is much nearer the value expected from the other eighteen determinations.

TABLE 1. Histamine changes after adrenalectomy

Tissue	No. of determinations	Mean histamine content in control rats μg./g. fresh tissue	Mean histamine content in operated rats μg./g. fresh tissue	Mean % difference log. calc.
Whole body	16	14·4	18·9	+31·1
Blood	28	0·99	1·47	+51·0
Whole gut	14	11·2	10·0	− 8·61
Stomach	21	35·2	36·2	− 1·17
Small intestine	15	6·30	10·4	+64·5
Caecum	9	15·0	22·8	+52·4
Large intestine	10	4·30	6·29	+42·1
Liver	19	7·72	77·4	+104·6
Kidney	18	1·86	2·34	+24·7
Spleen	19	2·65	4·64	+70·2
Striated muscle	15	8·7	10·3	+17·8
Cardiac muscle	19	5·5	7·0	+22·8
Skin	17	53·2	66·8	+26·9
Lung	38	5·4	6·5	+23·2
Brain	—	Histamine content too small for estimation		

Changes in water content of the tissues after adrenalectomy were found to be small, and only in blood, small intestine, liver, kidney and lung was the change significant. Of these tissues lung lost water after adrenalectomy, making the histamine increase *less* when calculated in terms of dry tissue, while the other tissues gained water, which *increased* the value of the histamine change when calculated in terms of dry tissue.

TABLE 2. Control experiments

Tissue	Mean tissue histamine μg./g.	Mean tissue +added histamine μg./g.	Mean difference (added histamine found)	Histamine actually added	Mean % histamine found	Mean % histamine added
Blood*	1·34	2·46	1·12	0·57	100·0	54·4
Blood†	0·41	0·75	0·35	0·52	88·1	161·7
Stomach	43·7	78·5	34·9	21·0	87·1	53·1
Small intestine	8·78	14·4	5·6	5·1	66·5	61·2
Caecum	13·0	21·6	8·6	7·8	65·4	60·3
Large intestine	11·7	13·5	1·8	2·0	15·9	18·7
Liver	2·43	4·55	2·21	1·95	94·4	86·5
Kidney	1·04	1·62	0·58	0·60	47·9	65·2
Spleen	3·09	3·68	0·58	1·45	32·0	62·1
Striated muscle	8·73	10·20	1·45	2·06	14·9	24·1
Cardiac muscle	4·28	4·49	0·21	1·49	12·7	41·1
Skin	40·5	57·9	17·3	15·1	40·4	40·2
Lung	4·38	7·50	3·11	1·53	75·6	37·5

* Method of Best & McHenry [1930].
† Code's [1937] modification of the method of Barsoum & Gaddum [1935].

The results of the control experiments are shown in Table 2. Control experiments on blood were carried out using both Code's [1937] modification of Barsoum & Gaddum's [1935] method for the extraction of histamine from blood, and the method of Best & McHenry [1930] for tissues. The mean percentage values given in the last two columns of the table were calculated by the logarithmic method in order to make them comparable with the values recorded in Table 1. Statistical analysis of these results showed that added histamine could be recovered with reasonable accuracy from all the tissues examined with the exception of blood, spleen, cardiac muscle and lung.

STATISTICAL ANALYSIS OF RESULTS

All the results obtained were analysed statistically to ascertain how far each mean value quoted could be relied upon. In each experiment the standard deviation (σ) of the mean value of n determinations was calculated. The standard error (E) was then calculated from the formula $E = \sigma/n$, and the value of t, the significance of the mean value (M), was obtained from the formula $t = M/E$.

In the experiments on tissue-histamine change after adrenalectomy, and those on change of water content in the tissues after adrenalectomy, t was calculated for the log mean differences between tissues from control and operated rats. In the control experiments the value of t was calculated for the log mean percentage of added histamine found, and also for the log mean difference between added histamine found and histamine actually added.

All values of t were compared with values of t for $(n-1)$ determinations in a table of probabilities published by Fisher [1925]. Where the calculated value of t is equivalent to a value for P (probability) of more than 0·05, the mean is considered not to be significant; where t is equivalent to a value for P of less than 0·05, the mean value of the series of results was considered significant.

For purposes of comparison Table 3 shows the results obtained for histamine changes after adrenalectomy by Wilson, Rose and Browne, and in the present work. To make the comparison more accurate, mean values from the figures reported by the other workers have been calculated by the logarithmic method described above, and the values so obtained subjected to statistical analysis. Reference to the table of results in Rose & Browne's paper shows that, in some experiments, there are unequal numbers of observations reported on control and operated rats. Since the logarithmic method of calculation admits only of comparisons between pairs of results, the 'odd' values had to be omitted. The calculation is also based on the assumption that pairs of values on the same line are comparable as controlled pairs, which is not stated in the text. The control values reported by Wilson were obtained from the same animals before adrenalectomy, and control and experimental values may therefore be safely regarded as strictly controlled pairs.

TABLE 3. Comparative table of results for histamine changes after adrenalectomy

S = Significant change; I = Insignificant change

| | Rose & Browne [1941] (rat) | | | | | | Present work (rat) | | |
| | Maintained on saline 11 days | | | Saline 7 days, water 4 days | | | | | |
Tissue	No. of determinations	% histamine change	Significance	No. of determinations	% histamine change	Significance	No. of determinations	% histamine change	Significance
Whole body	—	—	—	—	—	—	10	+31.1	S
Blood	—	Little change	—	—	—	—	28	+51.0	S
Whole gut	—	—	—	—	—	—	14	− 8.01	I
Stomach	12	+89.0	S	0	+202.4	S	21	− 1.17	I
Small intestine	12	+108.4	S	0	+231.3	S	15	+64.5	S
Caecum	—	—	—	—	—	—	9	+52.4	S
Large intestine	—	.	—	—	—	—	10	+42.1	S
Liver	5	+53.1	—	—	—	—	19	+104.6	S
Kidney	—	Little change	—	—	—	—	18	+24.7	S
Spleen	—	Little change	—	—	—	—	19	+70.2	S
Striated muscle	—	—	—	—	—	—	15	+17.8	S
Cardiac muscle	—	—	—	—	—	—	19	+22.8	S
Skin	—	—	—	—	—	—	19	+26.0	S
Lung	8	+25.8	I	—	—	—	17	+23.2	S

Wilson [1941] in six determinations on rabbits obtained a histamine change in blood of +108.4%. This is statistically significant.

Discussion

The results obtained in the experiments described are, generally speaking, in agreement with those of Wilson [1941] and of Rose & Browne [1941], in that there is in most of the tissues examined a significant increase in histamine content after adrenalectomy.

Rose & Browne [1941] suggested that the increase of histamine in the tissues of the adrenalectomized rat was due to transference of histamine from the blood, without any increase in the histamine content of the body as a whole. Wilson [1941], however, showed that the blood histamine was increased in adrenalectomized rabbits, this result being confirmed for the rat in this communication. It has also been shown in the experiments reported here that the histamine content of the whole body is increased by about 30% after adrenalectomy, representing an increase of over 200μg. histamine base in a 50 g. rat.

Since the greatest increases of histamine were found in certain regions of the gut (large and small intestine and caecum) and in the liver, which is directly connected with the gut by the portal system, it might be suggested that the extra histamine enters the body via the alimentary tract. No significant change was found in the histamine in the whole gut with its contents. It might be, then, that there is in the adrenal-deficient animal a transference of histamine within the gut, probably from the lumen into the wall of the intestine and so by the portal blood to the liver. Histamine which passes through the liver enters the general circulation and becomes more or less evenly distributed throughout the other tissues. This transference may be brought about by the changes in the osmotic balance of the body which are characteristic of adrenal cortical deficiency. On the other hand, histamine itself is capable of altering the permeability of cell membranes. Whether the osmotic changes of Addison's disease are the cause of the change in distribution of histamine or whether increased amounts of histamine (due to suppression of a specific anti-histamine function of the hormones of the adrenal cortex) are responsible for the disturbed osmotic balance between the tissues, still remains to be established.

Summary

1. The histamine content of the whole body, blood, whole gut (with contents), stomach, small intestine, caecum, large intestine, liver, kidney, spleen, striated muscle, cardiac muscle, skin and lung was determined in adrenalectomized rats in comparison with similar determinations on litter-mate, 'sham' operated controls.

2. With the exception of whole gut and stomach, all these tissues showed a significant increase in histamine content following adrenalectomy.

3. Control experiments involving the recovery of histamine added in known quantities to the various tissues showed that the experimental methods em-

ployed were reliable except probably in the case of lung, cardiac muscle, spleen and blood.

· 4. All the results recorded were analysed statistically before conclusions were drawn.

5. The significance of the results is discussed, with reference to the probable source of the extra histamine present in the adrenal cortical deficient animal.

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RATE OF REGENERATION OF PERIPHERAL NERVES IN MAN

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The rate of regeneration of peripheral nerves in man has been so universally accepted as about 1 mm. a day that the basis for and accuracy of this estimate have long remained unchallenged. There is no doubt that the rate is something of this order as compared with figures such as 0·1 or 10 mm. a day. But the subject is a complex one. There may be different rates for fibres of different types, and it is probable, even likely, that the various methods of estimation at our disposal will give results that are not even comparable. Is it true that regeneration progresses at a uniform rate throughout the length of a nerve? We know that there is an initial delay at the site of the lesion, but once this is past what grounds are there for believing that the new fibres progress at a steady rate until they reach their end-organs?. Although the experimental work of Gutmann, Guttmann, Medawar & Young [1942] has answered many such questions in the rabbit, no comparable work has been done in man. ·

The rate of advance of Tinel's sign has long been the most popular method of estimating the rates of regeneration, and Tinel himself [1917] gives the rate of 1–2 mm. a day. Dustin [1917], with moderately accurate data in ten cases, gives approximate rates of 2–4·5 mm. a day for median, 1·5–2 mm. a day for ulnar, and 4–5 mm. a day for radial lesions. Although Trotter & Davies [1909] and Bunnell & Boyes [1927] were not concerned with rates of regeneration as such, their data enable one to work out approximate rates from diagrams showing return of sensibility in the skin. Stopford's [1920] accurate observations on motor recovery may also be used for working out rates, and his data will be analysed in detail later in this paper. Marble, Hamlin & Watkins [1942] give rates for the ulnar, median and radial nerves, but their data are inadequate and their method of calculation simply division of the total distance covered by the time taken before 'regeneration was noted to be complete'. However, the subject has never been reviewed at all comprehensively, nor has there been any investigation of factors responsible for differences between rates estimated by different methods. For example, it has recently been shown

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experimentally [Gutmann *et al.* 1942] that the rate of regeneration after suture is different from that after axonotmesis;[1] this alone is sufficient to stimulate an inquiry into the possibility of similar differences being found in man.

All these observations, except those made by Trotter & Davies who produced experimental lesions in themselves, have been made on patients suffering from nerve injury. In lesions of this kind there are many variables which make the path of the investigator a thorny one, but provided that the clinical material is adequate and observations are recorded faithfully, clinical cases are the medium of choice for they present lesions of a magnitude that could never be permitted in deliberate experiment, and the variables are themselves of such importance as to merit investigation. Before considering clinical methods in detail it is necessary to mention two relevant experimental methods; the usefulness of the first is doubtful, that of the second possible but as yet unestablished.

EXPERIMENTAL METHODS

(a) *'Pinch' method* [Young & Medawar, 1940]. This method has proved to be of real value for *comparing* rates of regeneration after crushing the nerves of experimental animals or after various forms of suture. At an appropriate time after the infliction of the lesion the nerve is exposed, mobilized, and crushed with fine forceps at millimetre intervals working from the periphery towards the lesion. The first point at which the animal gives a reflex response under light anaesthesia provides a measure of the distance to which the fastest growing pain fibres have penetrated the peripheral stump at that time. Histological examination shows that the method is delicate. It must, however, be emphasized that it measures the rate of advance of the fastest growing fibres, and the vanguard certainly travels more quickly than the main company. The pinch method, besides being almost inapplicable in man, is of limited clinical interest because the quantity it measures is of minor functional significance. Much the same is true of Tinel's sign (see below) which is probably a less sensitive measure of a quantity of the same sort.

(b) *Histological examination of regenerating nerves in amputated limbs.* Very occasionally one meets a patient requiring amputation for a condition other than malignant disease or extensive infection, who willingly consents to a nerve in the part to be sacrificed being crushed or divided and sutured. If the small experimental operation and the amputation are timed properly it is possible to obtain a length of nerve that can be cut into segments and examined for the presence of new fibres. Investigations of this kind have been made in

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[1] There are three chief types of nerve injury [Seddon, 1942]. Neurotmesis: division of the whole thickness of a nerve. Axonotmesis: a lesion 'in continuity' causing interruption of the axons sufficiently severe to produce complete peripheral degeneration, but sparing more or less of the supporting structure of the nerve. Neurapraxia: paralysis unaccompanied by peripheral degeneration and recovering rapidly and completely. Here we are concerned only with the first two.

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seven cases and will be reported elsewhere by J. Z. Young and H. J. Seddon. We feel hardly justified in including the rates here, because the number of observations (eight) is too small to give data of statistical value.

CLINICAL METHODS

(a) As regenerating fibres grow down the peripheral stump of a damaged nerve they re-innervate muscles in turn according to the anatomical order of innervation. If the process of recovery is followed diligently, it is possible to obtain data for the construction of a graph showing the rate at which regeneration proceeds; this is a good method in practice and will be discussed in detail.

(b) If, in a number of patients suffering from lesions of the same nerve but at different levels, the time taken for the regenerating axons to cover the distance from the lesion to a given muscle be observed, then by plotting the readings on a graph it should be possible to get an estimate of the rate of motor recovery. This method has been employed with success in experimental work on small animals. We have not yet had opportunity in man for working out rates in this way for a large number of comparable lesions of one nerve is required, and as the method involves consideration of genetic differences (all the factors peculiar to an individual which may have some bearing on the rate at which a nerve regenerates) it can never, in animals or man, give results at all comparable in accuracy with those obtained from calculations based on serial innervation. We do not, therefore, propose to discuss it further.

(c) The advance of Tinel's sign is generally taken as indicating the progress of the axon tip, and in a few cases we have found it to move forward with striking regularity. As evidence of regeneration, Tinel's sign is of value only after suture (our experience amply confirms Stookey's [1919]), and we have therefore confined our observations on rates to those cases in which Tinel's sign has been observed after suture.

(d) Clinical observations on the rate of advance of returning sensibility. This is a promising method of investigation but by no means as simple as would appear at first sight.

(e) Rate of return of activity in sweat glands. The demonstration of sweating by Guttmann's method [1940] has made the investigation of sudomotor phenomena a comparatively straightforward procedure, and we hoped that our serial photographic records would show recovery of sweating advancing in an orderly fashion; but unfortunately the process is erratic and therefore quite useless for the estimation of rates of regeneration.

SERIAL RE-INNERVATION OF THE MUSCLES OF A LIMB

The process of recovery is a complicated one, but for present purposes may conveniently be divided into three stages: (a) the initial delay at the site of the lesion, (b) the period during which the axons are travelling down the nerve,

and (c) the time that elapses between the arrival of the fibres in muscle or skin and the re-establishment of function., Of the three, the last phase is undoubtedly the most complex, for it includes the time required for 'maturation' of the fibres, for regeneration or restitution of end-organs, and for the attainment of a certain density of innervation which is necessary before clinical recovery becomes manifest. But, to follow the reasoning of Gutmann *et al.* [1942, p. 14], one may consider the process more simply as (a) the initial delay at the scar, or latent period, and (b) the advance of functionally complete fibres; not as the rate of advance of minute axon tips which are immature, but the rate of progress of functionally complete regeneration of the nerve. Since the methods to be described (apart from the rate of advance of Tinel's sign) depend on progressive re-establishment of function, this conception of regeneration is not merely convenient but the most accurate, since it gives the rate of advance of that process which, occurring in the main body of regenerating fibres, has as its end-point the appearance of voluntary contraction or return of sensibility.

Application of the method requires three sets of data: (a) the level of the lesion, (b) the points of entry of motor branches, and (c) times of reappearance of voluntary contraction.

(a) It is desirable though not essential that the level of the lesion should be known in relation to a convenient bony landmark and this has been noted in all cases that we have explored or sutured.

(b) We must know also the points of entry of nerves into muscles in relation to convenient bony landmarks; it will then be possible to work out the distance to be covered by the regenerating axons in their journey from the lesion to the various muscles. It is obvious that the point of entry of the nerve is not the same thing as the final destination of individual fibres and their branches for, as Frohse & Fränkel [1913] have shown, a motor nerve arborizes in the most elaborate way before breaking up into its terminal filaments. But there are such wide variations in the level at which a motor nerve enters a muscle, to say nothing of possible multiplicity of branches, that all calculations are uncertain and complex, and little would be gained by embarking on the almost impossible task of reducing the intramuscular ramifications of a motor nerve to linear measurement; in any case, this factor is constantly present (though it is not necessarily always of the same magnitude in different muscles).

Points of entry of motor branches

Although these data have not been neglected by anatomists and clinicians, only Linell's [1921] measurements are sufficiently detailed to be of value for this work. His figures are for the upper limb alone and are based on the dissection of twenty-six subjects. Linell found a constant relation between the length of a limb and the distance of points of entry of motor branches from a bony landmark, and by working out ratios he obtained remarkably constant

figures for what he considered the average limb, i.e. one in which the distance between the tip of the acromion and the tip of the lateral epicondyle is 30·5 cm., and the distance between the lateral epicondyle and the styloid process of the radius 24·04 cm. (the 0·04 is a remarkable refinement). His figures, which do not include any for the intrinsic muscles of the hand, are given alongside those collected in the Department of Human Anatomy at Oxford.

Under the direction of Prof. Le Gros Clark, a number of dissections have been carried out by W. Bremner Highet and, later and on a more extensive scale, by B. Feinstein. They both found considerable variations in levels of entry of branches, even between the two sides of the same subject, and there was no

TABLE 1. Points of entry of motor branches into muscles

UPPER LIMB

All in relation to a line drawn horizontally through the lateral epicondyle: distances in cm.

	Feinstein	Linell	
Radial nerve:			
Triceps: Long head	18	19·2	
Medial head	12	11·24	Above
Lateral head	15	15·88	epicondyle
Brachioradialis	2	1·24	
Extensor carpi radialis longus	0	0	
Extensor carpi radialis brevis	3·5	6·10	
Extensor communis digitorum	9	7·5	
Extensor carpi ulnaris	10	7·5	
Extensor minimi digiti	9·5	7·5	Below
Abductor pollicis longus	11	—	epicondyle
Extensor pollicis longus	14·5	—	
Extensor pollicis brevis	14	—	
Extensor indicis	14	—	
Median nerve:			
Pronator teres	4·5	1·5 –2	
Flexor carpi radialis	6·5		
Flexor sublimis digitorum	7	2·76–5·05	
Flexor pollicis longus	11·5	—	
Flexor profundus digitorum	10·5	—	
Thenar muscles	33	—	
Ulnar nerve:			
Flexor carpi ulnaris	3·5	2·08–2·99	
Flexor profundus digitorum	6·5	5·54	
Hypothenar muscles	31	—	
First dorsal interosseus and adductor pollicis	34		

LOWER LIMB

All in relation to a line drawn through the head of the fibula: distances in cm.

Peroneal nerve:		
Biceps: short head	20	Above head of fibula
Peroneus longus	6	
Peroneus brevis	13	
Tibialis anterior	5	
Extensor digitorum longus	13	Below head of fibula
Extensor hallucis longus	15	
Extensor digitorum brevis	40	
Tibial nerve:		
Gastrocnemius	1	Above head of fibula
Tibialis posterior	8	
Flexor digitorum longus	14	
Flexor hallucis longus	20	Below head of fibula
Abductor hallucis	38	

relation between the levels of entry and the length of limbs in subjects of different adult stature. Dr Feinstein has done this work as part of a more comprehensive investigation of the anatomical features of somatic motor nerves, but he has permitted us to give the average figures (which include Highet's data) for the benefit of others who may be working on this same problem.

(c) Lastly, we must know the times at which contractions first appeared in the various muscles innervated by branches arising from the peripheral stump. This immediately introduces difficulties, for in the majority of cases it is neither possible nor desirable to keep patients in hospital or convalescent home for the greater part of the period of recovery; and it is not always possible to see patients with the frequency that we desire. Stopford saw his patients at monthly intervals which meant that there might be an error of two or three weeks. In most of our cases we have had to be content with a similar frequency of observation, though in selected cases observations were made every few days and in a number at intervals of one week.[1] When a complete muscle chart has been made (Table 2) it is possible to plot the points on a graph (Fig. 1) which shows the distance covered by the process of regeneration in

TABLE 2. Muscle chart from a case of radial axonotmesis

Date 1941 ...	28. iii	3. vi	23. vi	3. vii	10. vii	17. vii	31. vii	14. viii
Brachioradialis	0	1	1·5	2	2	2·5	3	3
E.C.Rad.L.	0	0	1	2	3	3	3	3
E.C.Rad.B.	0	0	0	0	0	0	2	2
Supinator	0	0	0	0	?	?	?	—
E.Com.D.	0	0	0	0	0	0	0	0
E.Min.D.	0	0	0	0	0	0	0	0
E.C.Uln.	0	0	0	0	0	0	0	0
Abd.P.L.	0	0	0	-0	0	0	0	0
E.P.L.	0	0	0	0	0	0	0	0
E.P.B.	0	0	0	0	0	0	0	0
E.Indic.	0	0	0	0	0	0	0	0

Date 1941 ...	4. ix	18. ix	2. x	9. x	30. x	27. xi	11. xii
Brachioradialis	3	4	4	4	5	5	5
E.C.Rad.L.	4	4	4	4	4	4·5	5
E.C.Rad.B.	2	3	4	4	4	4·5	5
Supinator	0	?	3	4	4	4	—
E.Com.D.	0	2	3	3	3·5	3·5	3·5
E.Min.D.	0	2	2	3	3·5	3·5	3·5
E.C.Uln.	0	0	0	2	3	3	3
Abd.P.L.	0	0	0	2	3	3	3
E.P.L.	0	0	0	0	1	3	2
E.P.B.	0	0	0	0	0	1	2
E.Indic.	0	0	0	0	1	1	2

This chart shows progressive recovery in anatomical order (see Fig. 5).

5 = normal power.
4 = power short of normal, but contraction against resistance.
3 = contraction against gravity, but not against resistance.
2 = contraction, but not against gravity.
1 = flicker.
0 = total paralysis.

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[1] The return of faradic excitability is of no value, since in most cases it is preceded by the return of voluntary power.
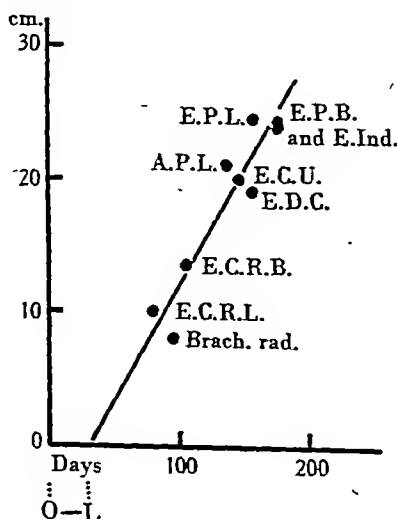
Fig. 1. Graph showing serial reinnervation of muscles in case LAM...;
radial axonotmesis. *O–L* is latent period.
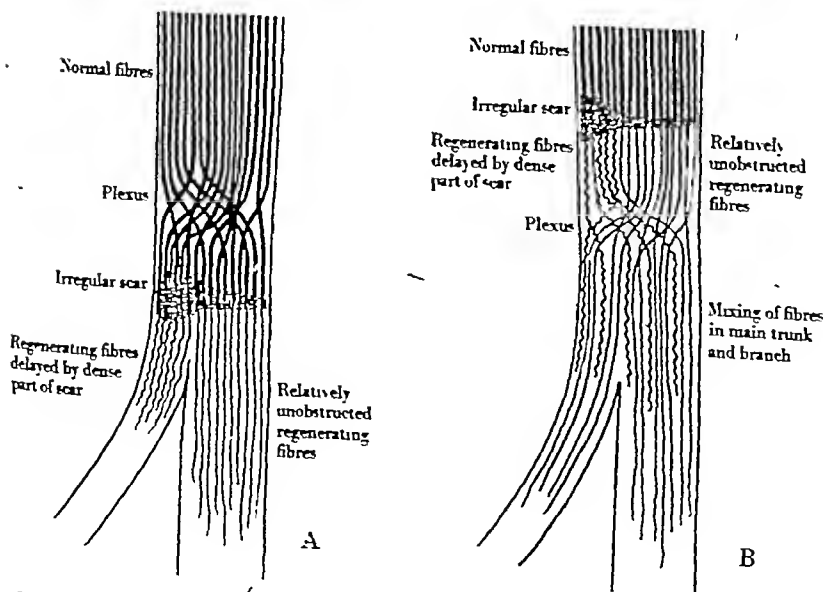
Fig. 2. Diagrams showing influence of intraneural plexus on regeneration in a case where the lesion is not of uniform severity. A shows delayed regeneration in a branch where the densest part of the scar is between the branch and the plexus. If the branch is to a muscle there will be undue delay in the return of voluntary power. B shows the mixing effect at the plexus when the lesion is central to it. In this case regeneration would be regular so far as return of voluntary power was concerned.

various motor nerves within certain periods of time. But even if we were quite certain that the axons travelled at a constant rate throughout, the points would not fall exactly along a regular line except by accident, because of anatomical variables already described and which cannot be eliminated. However, a coefficient of linear regression can be calculated in many cases and this gives some idea of the advance of regeneration of motor fibres, i.e. it gives an index of the rate of advance of the wave front of functional maturation rather than the rate of growth of the axon tips. When a line representing the growth rate is drawn so as to intersect the base line we should obtain a representation of the latent period ($O-L$, Fig. 1) or delay at the site of lesion.

Certain precautions must be taken both in the collection of data and in their interpretation. Lesions occurring in man rarely have the uniformity aimed at and often obtained in experimental lesions, and this is especially true in axonotmesis where we have frequently observed an asymmetry in the scar at the site of damage. When regeneration begins one can hardly expect the axons to advance on a uniform front; those that have little scar tissue to penetrate outstrip those confronted by more serious obstruction and, presumably, reach their destinations earlier, that is, if the same distance has to be covered. From this it follows that a lesion, whether axonotmesis or division followed by suture, only a short distance above the origin of branches will not be followed by regeneration in a completely regular order unless (and this is something of which we can rarely be sure) the lesion has affected the nerve uniformly throughout its thickness. Fig. 2 shows diagrammatically the sort of irregularity that is to be expected. However, if the lesion is well above the point at which branches first arise there will be a thorough mixing of fibres at the intraneural plexus proximal to the point at which branches arise [Foerster & Bumke, 1929; Kilvington, 1940]; by the time the fibres reach the first set of branches and the peripheral stump beyond, any irregularity at the site of the lesion will have become uniformly distributed. Our experience in the construction of graphs has compelled us to discard a number in which the lesion was close to the first set of branches.

MATERIAL

Out of 500 cases seen at the Oxford Centre, only 25 (including of course different nerves in the same patient) have been suitable for determination of rates by this method; and only in the case of the radial nerve has there been a sufficient number (18) to permit average rates being worked out.

RESULTS

Although estimation of rates of regeneration in the rabbit and the data from certain human cases, those represented in Fig. 1, for example, strongly suggest that the process advances at a uniform rate we are bound to consider another possibility.

Experience in other fields of biometry favours the probability of a progressive diminution in the rate; indeed, it is almost invariably found that the rate
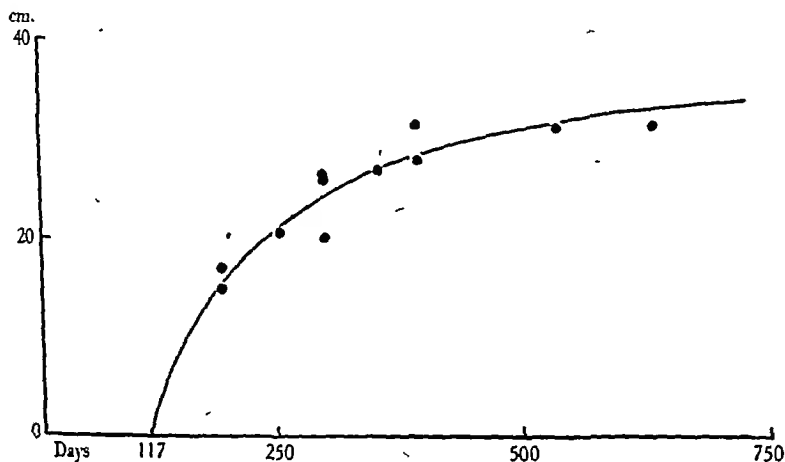
Fig. 3. Case BUT...; radial axonotmesis with slow motor recovery. A curve is clearly the best fit, but had the last two readings been omitted a straight line might have appeared equally satisfactory.
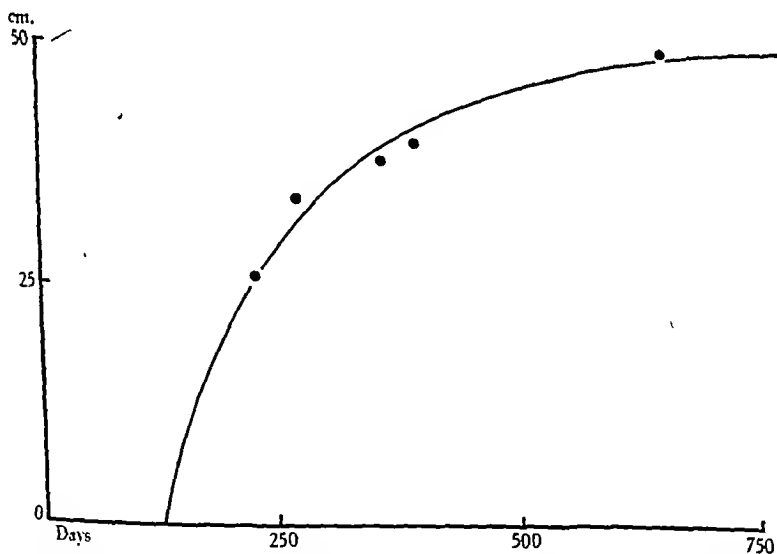
Fig. 4. Case LIN...; peroneal axonotmesis with sensory recovery observed over a long period. A curve gives the best fit.

of growth of anything, from a whole organism to a fragment of tissue, follows the same general tempo: it is fast at first and slows as the growth process approaches completion. Nevertheless the rate may be approximately constant

over moderately long periods, and unless the number of readings plotted on a graph is considerable the initial fast rate and the ultimate slowing may escape detection and the rate consequently appear to be capable of representation by a straight line. What is the general pattern of recovery in our cases?

Let us begin by considering two cases that were exceptional in that the periods of observation were unusually long. The first, BUT... (Fig. 3), was a patient who had a serious gunshot wound of the arm and complete paralysis of the radial nerve. The lesion must have been essentially an axonotmesis, since regeneration occurred spontaneously and progressed in an orderly fashion. But the points on the graph undoubtedly fall on to a curve: recovery was rapid at first, slow later. Nevertheless, it could fairly be represented by a straight line between the 194th and 300th days. The second case (which should properly be considered later under rates of return of sensibility) is of the same sort. The graph (Fig. 4) shows the rate of return of pain sensibility in the cutaneous area supplied by the peroneal nerve in a case of axonotmesis. A straight line cannot be fitted to these points. These are two outstanding

cases in which the progressive diminution in rate was immediately apparent; in other cases, including some of Stopford's, there was a less obvious but quite suggestive falling off in the later readings.

Coming now to our series of cases of axono- tmesis[1] and recovery after suture, we find twenty-three in which there was no difficulty in representing the rate of regeneration by a straight line. In the case ARN... (Fig. 5) the approximation is very close indeed, and in fifteen of Stopford's (1920) cases the same was possible (e.g. Fig. 6) though in one (no. 29) the data were poor and in three (nos. 5, 6 and 7) a curve was the obvious fit. (Stopford's data

Fig. 5. Case ARN...; radial axono-tmesis; motor recovery, showing negative latent period.

give remarkably good lines provided that the readings for extensor carpi ulnaris are omitted. He records this muscle as recovering in advance of extensor communis digitorum. This is not to be expected anatomically nor, in our

[1] Here it is necessary to remind the reader that the term 'axonotmesis' describes not an exact pathological entity so much as a whole series of conditions ranging from pure axonotmesis, in which the injury damaged only the axons leaving epi-, peri-, and endoneurium intact, to damage severe enough to break up the endoneural connective tissue to an extent only just short of complete division. The features common to all grades of axonotmesis are (1) that the nerve is in continuity, (2) that regeneration occurs spontaneously, and (3) that the quality of recovery is so good that one cannot but believe that most of the new fibres have grown along their old pathways. The variations in the extent of the damage are, however, revealed by the inconstancy of the delay before regeneration begins and perhaps by variations in the rate of regeneration.

experience, does it occur. And it is so difficult to be certain of a minimal contraction in this muscle that we feel justified in ignoring readings that are such obvious misfits.)

It is at once apparent that there are remarkable variations in rate, even for lesions of the same nerve and, if anything, even greater variations in the 'latent periods'. It may be possible, when more data are available, to explain these variations in terms of the severity of the damage and the intraneural scarring resulting from it. But it is important now to note that in seven of our cases the latent period, as calculated by extrapolation, falls on the negative side of the time axis. This is a manifest absurdity which forces one to the only possible conclusion that in these cases the process of regeneration progressed at more than the calculated rate during the early days. From this it follows that
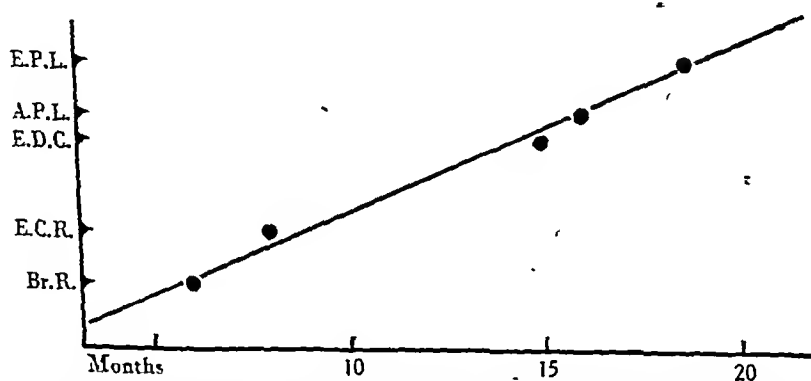
Fig. 6. .Graph of motor regeneration from data given for Stopford's case 8; radial suture. The latent period cannot be estimated since the exact level of the lesion was not given.

doubt must be thrown on the validity of the calculated latent period in those cases in which it is a positive quantity; the early rate in these cases may well have been faster too, and the true latent period longer than that calculated. It is clear, then, that although the curve of regeneration may approximate to a straight line over a very considerable period, there is evidence too weighty to be ignored that the process conforms more or less to that found in other fields, namely, that the rate of regeneration decreases as the process approaches completion.

Hence, the representation of the rate of regeneration by a straight line must be taken as nothing more than a sample of a curve the exact form of which can rarely be determined by direct observation. If this be borne in mind there will be no temptation to estimate the latent period by extrapolation. The linear 'rate', inexact as it is, has, however, some value in elucidating the behaviour of lesions of different types, and in the discussion of results the apparent linear rate will be used freely.

TABLE 3. Radial nerve: axonotmesis

Case	Mean rate mm./day	Lat. per. days	Mean dist. cm.
AND...	1·92	69	11·3
ARN...	0·96	− 15	21·8
BOS...	1·29	− 79	43·2
BUT...	Non-linear		
CRA...	1·36	25	23·0
CRO...	1·37	185	17·2
LAM...	1·72	29	18·2
MIT...	0·70	218	7·7
MOG...	1·64	− 40	44·5
PAV...	Non-linear		
PIC...	1·55	71	18·6
RIC...	2·09	103	16·9

Average: 1·46±0·13 mm./day.

TABLE 4. Radial nerve: suture

Case	Mean rate mm./day	Lat. per. days	Mean dist. cm.
FOG...	0·96	132	25·4
HIN...	2·42	151	11·9
MUL...	1·57	135	15·5
NAN...	1·61	68	9·6
RIC...	1·60	80	22·9
RUF...	1·26	155	6·6

Average: 1·57±0·20 mm./day.

TABLE 5. Radial nerve: suture (Stopford's cases)

Case		Rate mm./day	Case		Rate mm./day
1	Lower	0·49	27	Middle	0·45
2	Lower	0·74	28	Middle	0·50
5*	Lower	0·49	29†	Middle	1·03
6*	Lower	0·56	32	Middle	0·73
7*	Lower	0·58	41	Middle	0·45
8	Lower	0·41	52	Upper	0·73
9	Lower	0·59	53	Upper	0·40
26	Middle	0·73			

Average: 0·56±0·03 mm./day.

* Clearly curvilinear.   † Data poor; omitted in calculation.

TABLE 6. Other nerves

Case	Nerve	Lesion	Mean rate mm./day	Lat. per. days	Mean dist. cm.
PAT...	Ulnar	Axonotmesis	0·95	− 110	33·5
DOO...	Ulnar	Suture	1·33	52	27·8
HOL...	Ulnar	Suture	1·05	2	32·7
BAR...	Median	Axonotmesis	0·94	− 55	27·3
WEB...	Tibial	Axonotmesis	0·96	−218	49·8
SCA...	Peroneal	Axonotmesis	1·55	87	19·8
WEB...	Peroneal	Axonotmesis	1·38	− 48	49·5

Averages: all cases of axonotmesis 1·36±0·10 mm./da   ~~all cases of suture~~ (Oxford) 1·48±0·16 mm./day.
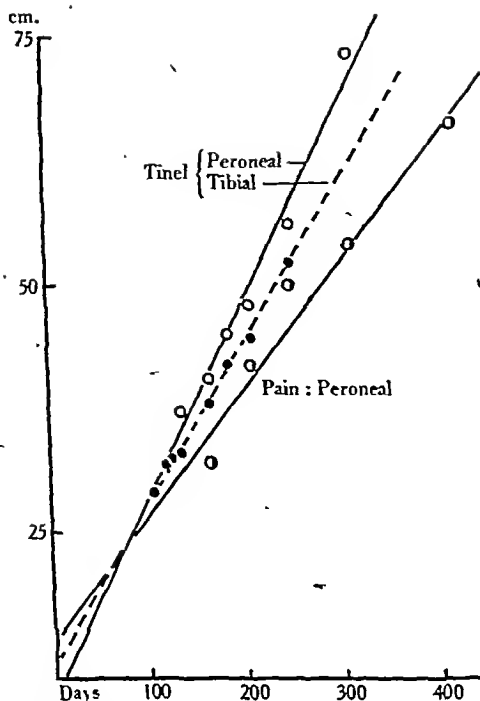
Fig. 7. Case BEN... (see also Fig. 9); sciatic suture. There is an appreciable difference between the rates of regeneration in the two divisions as calculated from the advance of Tinel's sign. Recovery of pain sensibility also shown. In each case the apparent latent period is a negative quantity.
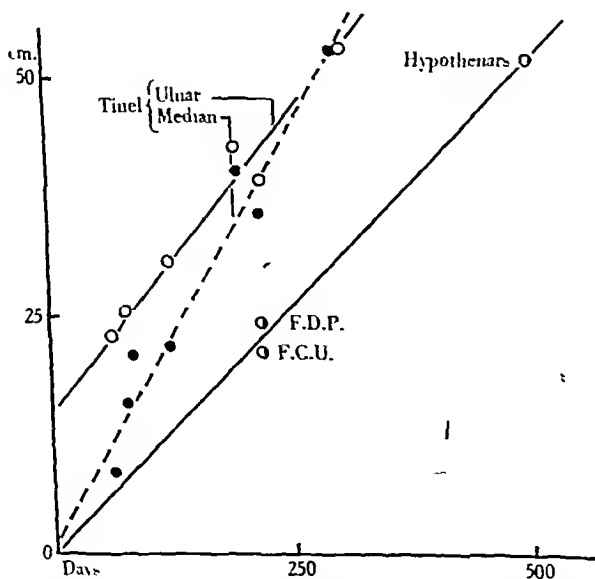
Fig. 8. Case HOL...; suture of median and ulnar nerves in the upper third of the arm. There is a difference between the rates of regeneration in the two nerves as calculated from the advance of Tinel's sign. Rate of motor recovery in the ulnar nerve also shown.

Regeneration as measured by Tinel's sign

This has long been a favourite method, and in a few cases where Tinel's sign advanced with notable regularity after nerve suture we have constructed graphs showing the rate of regeneration. The eliciting of Tinel's sign is the clinical equivalent of the experimental 'pinch' method of Young & Medawar [1940], only one would expect that a greater density of fibres would be required to give a response to tapping over the nerve than to give a response to a firm pinch of the exposed nerve: the wave front for Tinel's sign should therefore lag behind that for the pinch method, but be well in front of that for motor and sensory fibres that have advanced to functional maturity.

Progressive recovery of sensibility

The exact pattern of sensory recovery is still a controversial matter, but three well-defined phases are now known:

(*a*) Marginal shrinkage due to ingrowth of fibres from surrounding nerves [Pollock, 1920; Weddell, Guttmann & Gutmann, 1941].

(*b*) Regeneration in the damaged nerve manifest clinically as recovery by circumferential shrinkage, well seen in areas such as that supplied by the cutaneous part of the circumflex nerve.

(c) Progressive centrifugal recovery in those cases which show a long zone of sensory loss (Fig. 9).

Only the last is capable of fairly accurate measurement, and in our work only pain and touch have been followed. In one way the method is superior to that based on motor recovery, for the measurements made are accurate for the day of observation (whereas only the earliest contraction of a muscle is significant) and no reliance has to be placed on data obtained by dissection. The limiting factor is the number of cutaneous areas sufficiently large and, more especially, sufficiently elongated to

Fig. 9. Case BEN...; sciatic suture. Composite drawing made from serial photographs showing recovery of pain sensibility in peroneal and sural areas.

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29. ix. 41
12. i. 42

enable observations of this kind to be made over any length of time. But the examples given are sufficient to show that the method has its uses.

TABLE 7. Tinel's sign: suture

Case	Nerve	Mean rate mm./day	Lat. per. days
DOO...	Ulnar	2·25	3
FOL...	Ulnar	1·37	− 22
BEN... (Fig. 7)	(Peroneal	2·04	− 36
	(Tibial	1·59	− 76
HOL... (Fig. 8)	(Median	1·75	− 7
	(Ulnar	1·26	−119

Average rate: 1·71 mm./day.

TABLE 8. Recovery of sensibility

Modality	Case	Nerve	Mean rate mm./day	Lat. per. days
Pain	BEN...*	Peroneal	1·23	−110
Pain	BRO...	Peroneal	1·19	− 34
Pain	LIN...†	Peroneal	Non-linear	—
Touch	SED...	(Lateral cutaneous (of thigh	0·78	—

Average rate: 1·08 mm./day.

* Figs. 7 and 9. † Fig. 4.

These figures are too few to merit discussion, but they serve to show the sort of results that may be obtained by plotting the progress of recovery of sensibility.

DISCUSSION

Analysis of the data in motor recovery

The measurements between which a correlation has to be established are of two sets of quantities: the distances, from the site of the lesion, of the muscles innervated by a damaged nerve; and the corresponding times, measured from the date of the injury or of the operation, at which the muscles give their first signs of voluntary contraction. If these two sets of quantities prove to be related, as nearly as can be expected, by the curve of a continuous function, then it will be possible to express our results as a rate of advance of motor fibres of such maturity and in such number as to enable previously paralysed muscles to contract. The same applies in recovery of sensibility.

The general form of the curve of recovery

In most of our cases the assumption that the rate of recovery is *constant* over the range of times and distances within which the readings lie gives a very satisfactory approximation to the data. In about half the cases for which a straight line gives a good fit, however, the point of intersection of the line with the time-axis defines a negative quantity (Table 3 and Fig. 5); if the rate were constant throughout the whole period of recovery, then the point of intersection would define the latent period before recovery began. A negative latent period would imply that recovery began before the lesion was inflicted or repaired, which is clearly absurd. The point of intersection has, therefore, no literal meaning; and the inference is that the rate of recovery is higher in its earlier stages than it is within the range of times and distances accessible to measurement.

This conclusion is reinforced by the small number of cases (BUT..., Fig. 3; LIN..., Fig. 4; PAV... and certain of Stopford's) in which the progress of recovery was watched over a comparatively long period—little short of two years. In these cases, purely empirical curve-fitting confirms what is obvious to the naked eye, namely, that a curve which is steep at first and which flattens out progressively is the only one that can fit the data with reasonable accuracy. The exact form of this curve cannot be determined from data as meagre as ours are obliged to be; but in three cases which have been examined in detail, good fits are given by the assumption that the rate of recovery is inversely proportional to the square of the time (see Table 9). It should be emphasized,

TABLE 9

	Function	Equation: y = mm./day	Latent period days	Initial rate mm./day	V
LIN...	Logarithmic	$475{\cdot}6 \log t - 839{\cdot}4$	58	3·6	2·05
	Polynomial	$9 + 1{\cdot}409t - 0{\cdot}00103t^3$	− 6	1·4	1·72
	Monomolecular	$986{\cdot}9 - 908{\cdot}5\epsilon^{-0{\cdot}00107t}$	− 77	0·9	6·48
	Hyperbola	$601{\cdot}1 - \dfrac{76150}{t}$	127	4·7	1·24
PAV...	Logarithmic	$307{\cdot}7 \log t - 534{\cdot}5$	55	2·4	1·35
	Polynomial	$18{\cdot}4 + 0{\cdot}812t - 0{\cdot}0003857t^2$	− 22	0·8	1·38
	Monomolecular	$883{\cdot}2 - 866{\cdot}5\epsilon^{-0{\cdot}00093t}$	− 21	0 8	1·39
	Hyperbola	$313{\cdot}0 - \dfrac{25596}{t}$	81	2·6	1·58
BUT...	Logarithmic	$221{\cdot}8 \log t - 309{\cdot}8$	25	3·9	8·26
	Polynomial	$1{\cdot}4407t - 0{\cdot}001933t^2 - 70{\cdot}7$	53	1·3	3·32
	Hyperbola	$397 - \dfrac{46460}{t}$	117	3·4	3·95

however, that arcs of a great many curves can be made to fit the data adequately if the constants are suitably chosen. The table demonstrates the goodness of fit of four equations which have been widely used in studies on growth and regeneration. The third column sets out the value of t when $y = 0$, i.e. the estimated latent period t_0; and the fourth gives the hypothetical initial rate. The fifth column gives us a measure of the goodness of fit of each equation, i.e. the sum of the squares of the differences between the values predicted and those actually observed. The lower the value, the better the fit. The equations which give negative latent periods must be excluded; so likewise the polynomial in case BUT... which gives a maximum within the span of the readings. In each case the hyperbola gives an adequate fit, and predicts values for the latent periods and initial rates which are consistent with those which have been found in the rabbit (see below). The choice of this curve corresponds to the assumption that the rate of recovery is inversely proportional to the square of the time; but all we shall infer from it is that the rate of regeneration is initially as high as 3 mm. a day, and that it falls off progressively down to and then below a value of the order of 1 mm. a day about 100 days after recovery has started.

A third, indirect, method of testing the hypothesis that the rate of recovery falls off progressively is as follows: in each case for which a straight line gives an adequate fit to the data, the best estimate of the rate of recovery is that given by the slope of a line which pivots about the point $(\bar{t}, \bar{y})$, i.e. that determined by the mean time and the mean distance, and which is such that the sum of the squares of the differences between the values observed and those predicted by the equation of the line is minimal. If, however, the regression line is really curved, so that the straight line fitted to the array of data provided by each case is merely an approximation to that arc of the curve about which the points happen to lie, then it should be possible to find a significant decline in the estimated rates of recovery as the mean distances $\bar{y}$—i.e. the values of the points about which the lines pivot—increase. Alternatively, it should
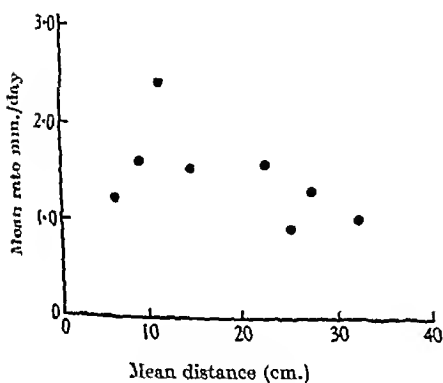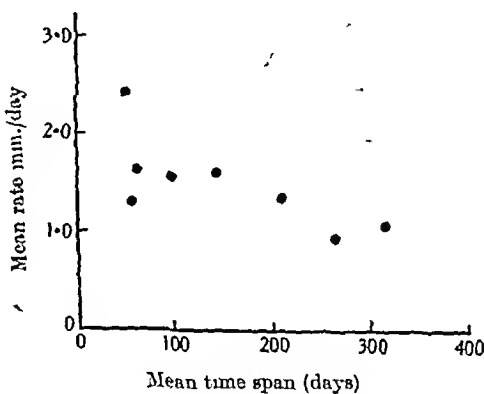
Fig. 10. See text. Fig. 11. See text.

be possible to establish a similar correlation between the rates calculated in each case, and the *mean time spans* $(\bar{t}-t_0)$ over which the readings have been taken.

The two correlation diagrams relating to the more or less homogeneous body of data provided by our estimates of motor recovery after suture are set out in Figs. 10 and 11. The correlation is not technically significant in the test illustrated by the former figure, in which the estimated rates are plotted against the mean distances. This is not surprising: the curve of recovery must be assumed to flatten sharply in the dimension of y, so that big differences between the estimated rates of recovery are associated with only small differences between the values of $\bar{y}$. On the other hand, when we plot the estimated rates against the mean time spans (Fig. 11), this objection does not arise; and we find that the chances are about 19:1 against the correlation observed being due to factors of sampling alone.

The data from Stopford's fifteen cases of motor recovery after radial nerve suture have been tested in a similar way. We have attempted to correlate the

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estimated rates with the *mean times*, and not with the quantities defined above as the 'mean time spans', because the exact level of the lesion was not specified by Stopford, and no value for the latent period (t_0) can be obtained. There can be no question of the significance of the decline in the rates as the mean time over which the readings were taken increases (Fig. 12); the chances are more than 100:1 against such a correlation being due to sampling factors alone.

One factor of which we know little may make the decline in rate appear more pronounced than it actually is. There is a progressive atrophy of muscle after denervation, and it is possible that the longer a muscle has been deprived of its nerve supply the longer will be the interval between the arrival of motor fibres in it and the appearance of the first voluntary contraction. Although in many cases the most significant atrophy has occurred before re-innervation begins, we cannot yet estimate its influence; but from work now in progress we suspect that it is not great in cases of the sort made use of here.

Fig. 12. See text.

To summarize: over moderate ranges of times and distance, the rates of recovery may be reasonably assumed to be constant. That this rate is not constant over the *whole* period of recovery, but falls off progressively, may be demonstrated in three ways: (a) by the fact that the assumption of a constant rate throughout leads in many cases to the manifest absurdity of accepting a negative value for the latent period; (b) by the fact that in the few cases where recovery has been watched for long periods (600 days or more) the line of regression is obviously curvilinear; and (c) by the fact that the rates, calculated on the assumption that they may be regarded as constant over moderate periods, become lower and lower as the mean periods over which the measurements were taken become further removed from the time when regeneration started.

Recovery rates in the rabbit and in man

In almost, though not quite, all of the data collected by Gutmann *et al.* [1942] for recovery rates in the rabbit, the assumption of a constant rate gave an excellent approximation throughout the *whole* of the time span over which the observations were made. Furthermore, the rates calculated on this assumption were much higher (2·5–4·5 mm. a day) than those calculated, using the same assumption, for man.

If we knew that the rabbit, so far as regeneration of nerve was concerned, behaved like man but on a smaller scale, then this discrepancy would almost
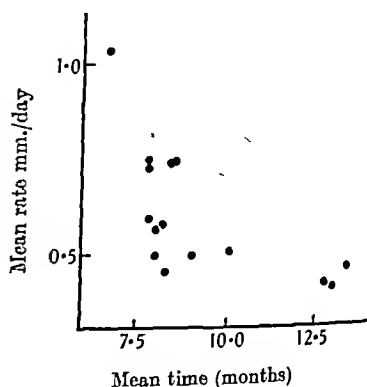
defy solution: But this is not likely to be the case; although it has never been proved, it is reasonable to suppose that mammalian nerve fibres of about the same order of diameter will behave similarly during regeneration, regardless of species. The work of Duncan [1934] on the size of motor fibres in the rat, the cat, and the cow shows conclusively that the diameter of fibres is not directly proportional to the size of the animal: on the contrary, the smaller the animal the larger the fibres relative to the total bulk. In other words, the rabbit's motor fibres are not likely to be very dissimilar in their behaviour from man's; and in regeneration the rates observed (being of necessity over a short distance —about 10 cm.—and close to the lesion) are comparable with those which the calculations suggest obtain in the *early stages* of regeneration in man (Table 9, column 4). The first part of the hypothetical curve in man is comparatively steep (Fig. 3). The rabbit has, so to speak, no time to develop a falling-off curve because its legs are not long enough.

It follows that measurements made in the rabbit can only be made to correspond to measurements of muscle recovery in man in cases where the muscles lie within 10 cm. of the point of the lesion. For technical reasons, no sufficient number of measurements within this narrow compass have been obtained in man; but the evidence we have analysed in the preceding section shows that in the earliest stages of recovery a rate of at least 3 mm. a day is to be expected. This is fully consistent with the more detailed evidence obtained from the rabbit. No doubt the somewhat longer latent periods we have predicted are connected with the greater severity of our lesions, and the more complicated nature of the function, voluntary muscle contraction, which we are studying [see Gutmann et al. 1942].

Rates of recovery after different types of lesion

From what has been said above, it follows that an expression for the *average* rate of recovery of some function after lesions of a nominally similar type could carry very little weight: our various estimates have been drawn from observations made over different spans of time and distance, and the lesions grouped together under the headings of *suture* and *axonotmesis* are certainly not as uniform among themselves as those inflicted experimentally on the rabbit.

However, there is no doubt whatever that the latent period after suture is longer than that after axonotmesis (Tables 3–6). There is also greater range of variation (about 300 days) in the calculated 'latent periods' after axonotmesis —see the ten cases of radial axonotmesis in Table 1—than after suture (about 90 days)—see the six cases in Table 4. This is in accordance with clinical and operative observations, for the extent of the damage to a nerve in axonotmesis varied greatly from one case to another [Seddon, unpublished work], whereas the local state of affairs after well-executed resection and secondary suture is a fairly constant one.

However, there are two variables in cases of suture too significant to be ignored: the time that elapses between injury and operation, and the severity of the stretching needed to lengthen a nerve after resection and suture in those cases which require flexion of a joint for satisfactory apposition. It is common knowledge that undue delay (for more than two years) diminishes the chances of successful recovery after suture. The reasons for this decline have not been fully elucidated though the important part played by the peripheral stump has recently been investigated by Holmes & Young [1942], who showed that there is a progressive shrinkage of Schwann tubes and decrease in the activity of the Schwann cells. The decline is almost certainly gradual, and it may well be that in a series of cases in which the only variable is the time elapsing between injury and suture a corresponding decrement of rate of regeneration (and quality) would be found. Post-operative stretching, if considerable, can be so noxious as to make regeneration impossible: it may produce intraneural damage of even greater extent than that resulting from the original injury [Highet & Holmes, 1943; Highet & Sanders, 1943]. This imposes a limitation on what can be accomplished after suture of a nerve where there has been considerable loss of substance. We know that excessive stretching may produce a complete bar to regeneration. It is likely, then, that lesser degrees of stretching will produce lesser, but perceptible, effects on the process of regeneration. These effects might be revealed in three ways: (a) by an increase in the latent period, (b) by a slowing of the rate of regeneration, and (c) by the poor quality of functional recovery. The first two possibilities could be investigated by any of the methods described for calculating rates of regeneration. That there is no correlation between the rates and latent periods after axonotmesis is of little significance. It is not difficult to believe that after axonotmesis the rate of regeneration has little to do with the time taken for fibres to traverse the scar at the site of injury. But the changes after stretching of a sutured nerve can be *very widespread* and are known to have a deleterious effect in reaches of the nerve well remote from the suture line.

In rabbits [Gutmann *et al.* 1942] there is a significant difference in the rates of recovery after crushing a nerve (axonotmesis) as compared with those after suture; after crushing, the axon tip advances at $4\cdot36\pm0\cdot24$ mm. a day, after suture at $3\cdot45\pm0\cdot16$; after crushing, functional completion advances at $3\cdot05\pm0\cdot14$ mm. a day, after suture at $2\cdot0\pm0\cdot3$. No similar difference has been found in our series of cases, and that observed by Sharpey Schafer [1927] in the experiment performed on himself must be discounted since the divided nerve was not sutured [Norman Dott, personal communication]. There are two possible explanations for these findings. Axonotmesis, which in the strict sense means a break in the axons without severance of any supporting structures, is known for certain only as an experimental lesion, produced by firm crushing of a nerve with fine smooth-bladed forceps; and it is followed by

regeneration of fibres into their old channels with a minimum of multiple sprouting from the central ends of the nerve fibres. Many cases of axonotmesis seen clinically show evidence of greater intraneural disturbance; and indeed if care is not exercised in the production of the experimental lesion a certain amount of damage to supporting structures with consequent funicular disruption may occur. So that although clinical cases of axonotmesis present certain features in common (continuity of the nerve, spontaneous regeneration, and recovery of such excellent quality that one must suppose that most fibres have in fact regained their old paths) the local disturbance may well be such that, when regeneration begins, the axons send out many sprouts in the way they invariably do after suture. This state of affairs has been seen after experimental lesions in man [Young & Seddon, unpublished work]. If the rate of regeneration is an expression of a protoplasmic outflow (which we now regard as an essential feature of regeneration of nerve) then there is no reason to anticipate any difference between the rates after axonotmesis with severe damage and suture, since the behaviour of the axon tips is similar in both. On the other hand, 'pure' axonotmesis (such as occurs in man after compression of a nerve) may well show a faster rate of regeneration since the regenerating fibre is not wasting its substance in useless branching. This question can only be settled by further observation.

Another explanation may be that after a time (perhaps after atrophy of the adventitious and abortive sprouts thrown out in the early stages of regeneration after suture) the rate of regeneration after suture may become equal to that after axonotmesis. As has already been explained, observations as late as those recorded in clinical cases cannot be made in the rabbit, and the process is probably complete before there is time for any approximation of rates so manifestly different in the early stages.

As soon, therefore, as sufficient material is available we propose to find out if there is any correlation between the loss of substance after secondary suture (and the post-operative stretching is roughly proportionate to the size of gap) on the one hand, and the rate of regeneration and latent period on the other. The difficulty, which can only be overcome by the collection and analysis of a large volume of material, is that the two variables, delay before suture and length lost as a result of resection, occur in every case except primary suture, when both are absent.

The variations in rate shown in the tables, so far from being discouraging, are to be regarded rather as adumbrations of further knowledge of the behaviour of nerves after injury that may well be of value in clinical practice.

There is a curious difference between the rates in Stopford's cases of suture of the radial nerve and those in our cases. The average for his cases (Table 5) is $0 \cdot 56 \pm 0 \cdot 03$ mm. a day, for ours (Table 4) $1 \cdot 57 \pm 0 \cdot 20$ mm. a day. Stopford [personal communication] believes that the cases in his series were operated

on at times after primary injury corresponding fairly closely with ours—about eight months—and this very striking discrepancy must for the present remain unexplained.

And there are other questions. Does regeneration proceed faster in young people? In a case of ulnar nerve suture in a boy of 16 (not included in the tables) the motor rate was at least 3 mm. a day. Are there different rates for different nerves? In WEB... (Table 6) a peroneal axonotmesis recovered at a motor rate of 1·38 mm. a day, the tibial axonotmesis at 0·96 mm. a day. In BEN... (Table 7, Fig. 7) Tinel's sign proceeded with remarkable regularity and the rates for the two divisions of the sciatic nerve were significantly different. And yet the lesion was of the main sciatic trunk and conditions at the suture line identical for both components. There is a similar discrepancy in HOL... (Table 7, Fig. 8), a case of suture of median and ulnar nerves in the upper third of the arm, carried out under very good conditions one month after the original injury. In this case there is the further difficulty that the motor rate was almost as fast as that for Tinel's sign.

Clinical applications

Neurolysis.[1] A favourite operation for axonotmesis is neurolysis, and many recoveries have been placed to its credit. In delayed traumatic ulnar neuritis this claim is justified because one removes the nerve from an environment which in the course of months or years was responsible for the gradual onset of degenerative changes. But in acute lesions the position is different. The nerve is damaged at the time of the accident; later, at operation, it is found embedded in scar tissue. To what extent is this scar tissue a bar to regeneration? Has the nerve not only been crushed by the first violence, but prevented from regenerating by an encircling collar or sheath of fibrous tissue, the release of which would permit the growth of fibres into the peripheral segment of the nerve? The findings at operation are sometimes impressive, the scar tissue is most extensive and the nerve appears to be strangulated. It is also remarkable, in cases where operations are performed for other conditions in which the nerve trunks of a limb are functionally normal, how well a nerve tolerates encasement in the dense scar tissue seen during the course of the operation. And there are several cases in our series where exploration revealed a damaged nerve in the most unfavourable surroundings and yet bipolar faradic stimulation produced contraction in muscles which, clinically, were completely paralysed; regeneration had already begun in spite of the burden of scar tissue or burial beneath callus. We thought it wise, therefore, in cases of axonotmesis not to attribute recovery to neurolysis unless it could be shown

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[1] We refer here only to external neurolysis, not to the irrational operation of removing scar tissue from the interior of a nerve trunk or the longitudinal incision of the damaged segment 'to relieve tension'.

beyond doubt by the calculated rate of regeneration that the process bore some sort of relation in time to the performance of the operation. Even if such relation were found, and the operation had been performed fairly soon after the injury, the beginning of recovery might still be coincidental. Judged by this criterion, no single case of axonotmesis has benefited from neurolysis or, as we now prefer to call the operation, exploration: by this criterion recovery was spontaneous. This test may perhaps be too rigid, but on the other hand the claims made for the operation have been quite fantastic in face of the known tendency of axonotmesis to recover spontaneously. As Platt [1924] says: 'It is difficult to prove that the process of regeneration per se is delayed or even completely retarded by its (perineural fibrous tissue) continued existence. It is still more difficult to prove that, after the removal of such external scarring, the occurrence of recovery is necessarily due to the operation alone.'

Prognosis. To the clinician, some knowledge of rates of regeneration is of great importance. If there are grounds for suspecting that recovery should occur spontaneously (e.g. in closed fractures of the humerus, radial paralysis is generally due to axono- rather than neurotmesis) the first sign of recovery— a flicker in brachioradialis—should be expected within a certain period. Even with closed lesions, it is usually not difficult to make a rough estimate of the level of damage; the point of entry of the nerve into the most proximal muscle is known (see p. 195); and the rate of regeneration is about 1·4 mm. a day. A simple calculation gives the estimated time for the appearance of the first flicker, and one may add, say, six or eight weeks for an unduly long latent period or a rate of regeneration that is below the average. If there is then no sign of recovery exploration should be considered, for the nerve may, in fact, be divided in which case further delay is time wasted. It sometimes happens that one suspects the soundness of a nerve suture: something may be known to have been faulty (wound infection, too rapid post-operative stretching), or the operation may have been performed elsewhere under conditions that did not make for success. Will recovery occur? Here again a simple calculation will give some indication of the time that must elapse before the first sign of recovery is to be expected; the rate is much the same as after axonotmesis, but a bigger allowance should be made, say two to three months, for the latent period.

Summary

1. The paper describes observations on the rates of recovery of sensory and motor functions in man after peripheral nerve lesions of different types.

2. After the interruption of a nerve, the muscles normally supplied by the part that lies peripheral to the lesion return to functioning in the anatomical order of their nerve supply. An estimate of the rate of motor recovery may be obtained by recording the times at which the muscles show their first signs of

voluntary contraction, and by making use of specially collected anatomical data showing the distances at which they lie from the level of the interruption.

3. The rate of sensory recovery may be calculated by measuring the advance of pain and touch sense in a long zone of cutaneous insensibility.

4. The rate of advance of Tinel's sign, though of limited functional significance, has some prognostic value and provides an additional method for the study of sensory recovery.

5. The rate of recovery falls off progressively as the process moves towards completion. It can however be regarded as constant over the moderate ranges of time and distance over which the process was recorded in the great majority of the cases described. The following average estimates have been obtained for the rate of motor recovery. In the radial nerve: after suture $1·6 \pm 0·2$ mm. day; after axonotmesis $1·5 \pm 0·1$ mm. day. In all nerves studied: after suture $1·5 \pm 0·2$ mm. day; after axonotmesis $1·4 \pm 0·1$ mm. day. The average rate of advance of Tinel's sign after suture has been found to be $1·7$ mm. day. Other estimates of sensory and motor recovery have been calculated from less complete data of our own and from the data of Stopford.

6. The principal variables that affect clinical estimates of the rate of recovery are, after suture: the interval between injury and repair of the nerve, the state of the stumps at the suture line, and the amount of damage inflicted by post-operative stretching in cases where extensive resection before suture had proved to be necessary. After axonotmesis: the extent of the damage to supporting structures within the trunk of the nerve.

7. When estimates have been corrected for the greater range of times and distances over which recovery in human cases can be recorded, the rates of recovery in man are not found to be greatly different from those estimated by more precise methods in the rabbit.

8. Studies of the rate of recovery in man are of value for the prognosis of nerve injuries and for judging the efficiency of the various types of operation used for their repair.

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REFLEX VASO-MOTOR RESPONSES OF
THE PAW OF THE CAT

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Hallion & Conte, as early as 1894, using plethysmographs, recorded on a smoked drum the volume changes occurring in the fingers and feet. They showed that a decrease of volume followed a nocuous stimulus or a deep breath and concluded that these changes represented active reflex vaso-constriction. Since that time many records have appeared in the literature, but it is only in recent years that observers have introduced more sensitive methods of recording volume changes. Information about the methods which have been introduced can be obtained from papers by Goetz [1935], Stürup, Bolton, Williams & Carmichael [1935] and Hertzman [1937].

In the following investigations a sensitive method has been used similar to that described by Stürup *et al.* [1935] to record the changes in the paw volume of cats. Small changes of volume are easily recorded and the time relations of the change accurately charted. We consider that the method will give much information of the mechanisms of vaso-constriction both at the periphery and at the reflex centres. A short summary of this work has been published previously [Downman, Goggio, McSwiney & Young, 1939].

METHOD

Glass plethysmographs of about 50 c.c. capacity were applied over the paws of the cat, reaching up to the wrist joint of the front paw and over the greater part of the metatarsus of the hind paw. The plethysmographs were connected to a brass capsule with rubber tubing, a side tube controlled by a spring clip allowing for equalization of pressure when necessary. A light plane mirror of polished silvered glass was mounted eccentrically upon a thin rubber membrane covering the end of the capsule. A beam of light was projected through a vertical slit on to the mirror and the movements of the reflected beam were recorded on photosensitive paper.

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The sensitivity of the system could be varied within wide limits by using rubber membranes of differing thickness on the capsules. It was most convenient to use a membrane which gave a deflexion equal to the width of the camera slit (58 mm.) for a volume change of 0·03–0·05 c.c. A less sensitive membrane would not record very small changes of volume; with a more sensitive membrane the deflexions due to each heart beat became inconveniently large.

Systemic blood pressure was recorded optically from the central cut end of a carotid artery. The arterial cannula was connected to a metal capsule carrying a thick rubber membrane with a stainless steel mirror attached; the mirror deflected the beam of light on to the camera slit. The system was filled with normal saline containing carrageen as anti-coagulant [Elsner, Braser & Bürgel, 1937; Elsner, 1938; Little, personal communication].

Respiratory movements were recorded optically by means of a rubber balloon held in contact with the epigastrium.

Typical calibration figures were of the following order:

Volume of glass plethysmograph	55 c.c.
Volume of connecting tube and capsule	11 c.c.
	66 c.c.
Volume of tissue in plethysmograph	30 c.c. (hind paw of 2·7 kg. cat)
Dead space	36 c.c.

The volume of tissue enclosed varied with the size of the animal, but was of the same order of magnitude for all animals.

Plethysmographs shaped to the form of tissue under investigation were also made by the following method. X-ray films were cleaned in hot water and the base dissolved in acetone to form a viscous solution. A layer of artificial silk-stocking material was applied to a wax model of the tissue and then painted over with the solution. When dry, alternate layers of fabric and solution were applied until a wall 2–3 mm. thick had been built up; the wax was scooped out and a glass side tube was cemented into a hole bored through the wall. The resulting structure was very rigid and withstood boiling. This method is rapid and can be adapted to produce any desired shape.

ANAESTHESIA

In the majority of experiments urethane (1·5 g./kg. body weight), nembutal (80 mg./kg.), or pernocton (0·4 c.c. 10 % solution per kg.) were used to produce anaesthesia. In a few cases anaesthesia was maintained with chloralose or dial (Ciba).

Urethane was administered intravenously or subcutaneously. In the former case preliminary ether anaesthesia was given, and the trachea cannulated. The anaesthetic was made up in normal saline as a 20 % solution. It was

seldom possible to administer the full dose in less than 30 min. owing to respiratory difficulties attendant upon a faster rate of administration; at a later stage in the experiments the respiratory rate was often very high, reaching 120 per min. in some animals. Reduction of the dose produced satisfactory anaesthesia but good vaso-motor responses were not obtained from the paws. In some animals responses were obtainable after about 5 hr. had elapsed from the intravenous injection of urethane. After a further period of about 2 hr. sensitivity was usually lost, and did not return. The respiratory difficulties consequent upon the use of intravenous urethane could generally be avoided by giving the same dose subcutaneously. We found that these animals were more sensitive to weak afferent stimulation than were those in which urethane had been given intravenously. The disadvantage of this method was that it was generally necessary to wait for 10–14 hr. before a response could be elicited; the period of sensitivity lasted slightly longer than in the case of intravenous urethane.

· Nembutal was dissolved in normal saline and administered intraperitoneally; deep anaesthesia was obtained within 10 min. In some animals reflex responses were obtained within 1 hour, but in others it was necessary to wait up to 3 hr. The animals were sensitive to weak afferent stimulation, and once sensitivity had appeared it did not pass off within the time required for the longer experiments (about 8–10 hr.), though in some animals anaesthesia became too light towards the end of this period.

Pernocton (supplied in 10 % solution) was diluted with 5–10 times its volume of normal saline and administered intravenously under ether anaesthesia. A slow rate of injection (2 c.c. diluted solution per minute) avoided difficulties with respiration. Reflex responses were usually obtainable within 2 hr. of administration of the anaesthetic; after a further variable time it was usually found that anaesthesia became too light, and the reflex responses were lost. In these circumstances a second injection of pernocton (equal to one-quarter of the original dose) generally restored both the required depth of anaesthesia and the reflex responses.

Positive results were also obtained with chloralose and dial anaesthesia. The chloralose (75–80 mg./kg. body weight) was injected as an approximately 2 % solution in saline into the femoral vein exposed under preliminary ether anaesthesia. Dial (Ciba) 0·65–0·70 ml./kg. was injected intraperitoneally. In our hands, however, these anaesthetics were not as reliable as the others, few of the animals remaining sensitive over long periods.

On the whole pernocton gave the most satisfactory results, the depth of anaesthesia being more easily controlled than when using urethane or nembutal. The use of nembutal had the advantage of dispensing with preliminary ether anaesthesia and avoided the possibility of the accumulation of mucus in the respiratory tract.

Results

Sensory stimulation caused a sharp decrease of volume of the paws of an-aesthetized and of decerebrate cats. The decrease of volume reached its maximum in a short interval of time and was followed by a slow return to the initial volume. In a sensitive animal the responses of the front and hind paws were synchronous. In some animals, however, the responses were limited to the hind paws, the front paws showing no change of volume. The decrease of volume of the paw was accompanied by a diminution of the amplitude of the pulsations, the return to the initial volume by an increase of amplitude up to its original size.
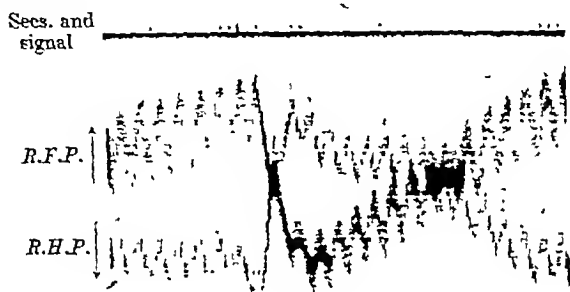
Fig. 1. Change of volume of front and hind paws following pinch of central end of cut left radial nerve. The smaller regular waves correspond with respirations. Urethane anaesthesia. Pinch at signal. *R.F.P.*=right front paw; *R.H.P.*=right hind paw. Arrows show direction of increase of volume. Time in seconds.

The volume change could be elicited by many types of stimulus, such as a noise, pinching the skin, irritation of exposed nerves, manipulation of the viscera; flicking the pinna with the finger was found to be a good test for sensitivity. In spite of the variety of stimuli there was no difference in the form or time relations of the responses.

Nature of the response

The diminution of volume is not caused by reflex movements; similar changes were obtained in the curarized decerebrate animal. The response could have been due to increased circulatory adrenaline, but this explanation cannot be accepted, because the latent period was too short, the decrease of paw volume after intravenous injection of adrenaline never taking place in less than 7·5 sec. Furthermore, the removal of both adrenal glands did not alter the response in magnitude or time relations.

The response is not secondary to alterations of blood flow to the limb, because clamping of the common iliac artery, though modifying the response,

did not prevent its occurrence in the ipsilateral hind paw. The volume change is also independent of alterations of systemic blood pressure. It usually preceded the recorded pressure changes in time, and was not apparently influenced by the direction of the change. In some animals it was accompanied by a rise, in others (especially under urethane) by a fall, and in some experiments there was no apparent change of blood pressure in the carotid artery.
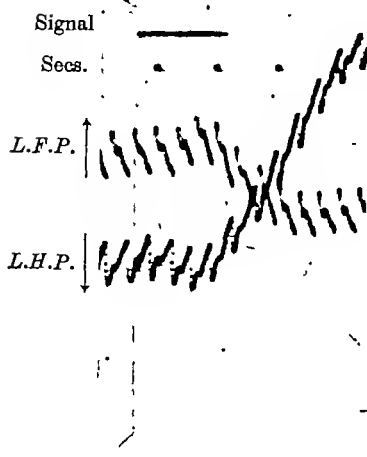
Fig. 2.
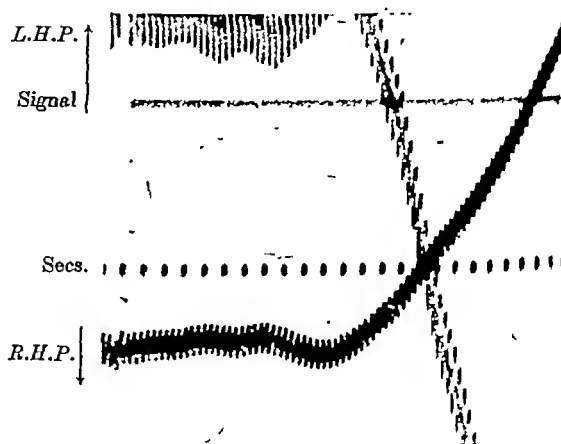
Fig. 3.

Fig. 2. Volume changes of left front and hind paws taken on more rapidly moving paper to show length of latent period. At beginning of signal line the central end of the cut radial nerve was stimulated by a single break induced shock, using a Lucas pendulum. Pernocton anaesthesia. Time in seconds.

Fig. 3. Volume changes of hind paws following injection of 1 μg. adrenaline in 1 ml. saline into left radial vein during signal. Pernocton anaesthesia. Time in seconds. Smaller pulses of right paw are due to use of stiffer rubber membrane carrying mirror.

A tight rubber ligature round the paw immediately proximal to the pads abolished the change of paw volume. This may be explained by the arrest of the circulation through the pads by the mechanical effect of the ligature. The abolition of the response is unlikely to be due to local damage to nervous paths, because release of the ligature was followed by its immediate return. If the pad of one digit was left out of the ligature the response was not abolished, showing that the previous results were not due to inhibition of nervous centres by the nocuous stimulus.

It is concluded, therefore, that the change of paw volume represents an active constriction of the vessels of the pads. Other possible causes, such as movement, changes of blood flow to the limb, and activity of the adrenal glands, have been controlled.

Features of the afferent side of the reflex arc

The wide range of effective stimuli has been referred to. A flick with the finger or a pinch of the pinna, pinching the skin of the body and loud, sharp sounds were effective. When using a Lucas pendulum it was necessary to remove it to another room as the sound of the fall of the arm was an effective stimulus to sensitive animals. Centripetal stimulation of the cut radial, saphenous, sciatic, vagus or splanchnic nerves was effective. The nerves were stimulated by pinching, faradization, or by single break induction shocks using the Lucas pendulum. The vagus was stimulated in the neck and the splanchnic nerves below the diaphragm. The response was also elicited by pulling on the root of the mesentery, distending a balloon in the pylorus (pressure 50–80 mm. Hg) or by pinching the small intestine.

When a graded series of induction shocks was given to an afferent nerve, increasing the current strength caused the decrease of paw volume to become maximal, but the blood pressure might fall, rise or show no change. Further increase of current strength caused no greater decrease of paw volume, but in some animals a previous fall of blood pressure was converted to a rise, or an existing rise augmented.

In a series of experiments the response was elicited by stimulation of the central cut end of a cutaneous nerve with weak faradic current. Increasing the duration of the stimulus up to approximately 60 sec. made no difference to the duration of the response, return to initial volume occurring during stimulation. When the strength of the faradic current was increased above a limiting value an increase in the duration of the stimulus now caused increased duration of the response, paw volume not returning to its base line until after the cessation of the stimulus.

Spinal nerve roots were exposed in the lumbar region by laminectomy. Single break shocks, faradization and pinching the central cut end of a dorsal root caused decrease of volume of both hind paws. Stimulation of the peripheral cut end of the root did not produce a response.

It is seen that the reflex can be elicited by stimulation of afferent nerves and end-organs situated in many different parts of the body.

Efferent pathways for the reflex

Section of the sympathetic pathway to the forelimb, either by removal of the stellate ganglion or section of the thoracic sympathetic chain proximal to the ganglion, abolished the response of the ipsilateral front paw. Stimulation of the distal cut end of the chain or pinching the stellate ganglion produced a response of the paw. Removal of both 4th lumbar sympathetic ganglia abolished the responses of both hind paws. Stimulation of the distal cut end

of either chain below this level caused decrease of volume of both hind paws, after the spinal cord of the lumbar region had been destroyed.

Stimulation of the distal cut end of a mixed nerve supplying the limb also caused responses of the corresponding paw in the curarized decerebrate cat.

Pinching the first, second or third anterior spinal roots of the lumbar region elicited a decrease of volume of the ipsilateral hind paw. The response persisted when the posterior roots were severed, and also when the corresponding segment of the cord was isolated and split in the mid-line. These results show that the impulses pass centrifugally, and that the response is not set up by impulses passing back into the cord.
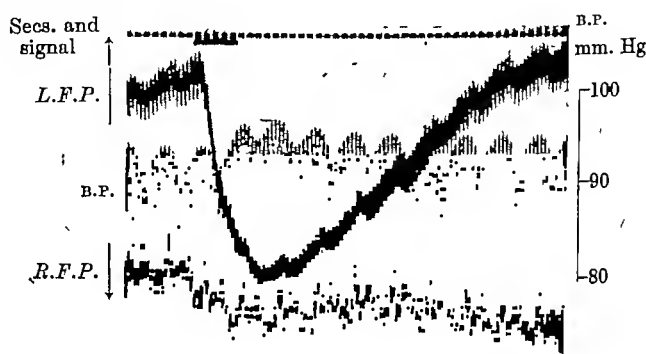
Fig. 4. Abolition of volume change by removal of right stellate ganglion. At signal faradization of central end of cut left saphenous nerve. Volume of front paws: blood pressure recorded from central end of cut left carotid artery. Time in seconds. Urethane anaesthesia.

It is concluded that the reflex vaso-constriction of the pads is mediated on the efferent side through the sympathetic system. The fibres leave the cord in the anterior roots, pass into the sympathetic chains and reach the pads after travelling along the mixed nerve trunks. Precise definition of the course and relay stations of the fibres was not attempted. The absence of a response to stimulation of the peripheral cut end of a dorsal nerve root suggests that there is no efferent pathway mediating vaso-constrictor reflexes of the paws along the sensory nerves. At the same time no vaso-dilatation was produced but this may have been due to the vessels being already maximally dilated.

Time relations of the response

In our experiments the reflex response had a latent period of 0·5–2·5 sec. Considerable variations were noted from time to time in the same animal on stimulation of the same afferent nerve, these variations depending mainly upon altered conditions of the peripheral blood vessels. It was observed that alteration of the latent period by as much as 0·5 sec. paralleled changes in tone of the vessels, the latent period being shorter if the vessels were con-

tricting at the time of the stimulus and longer if they were dilating. In a typical experiment the latent period was 0·67 sec. when the vessels were contricting and 1·12 sec. when they were dilating; these figures are averages of several observations taken over a short period when little variation occurred in each group. The latent period was increased by deepening the anaesthesia or by exhibiting curarine. Following the latent period the paw volume decreased rapidly, reaching a minimum in 2·5–10 sec. The time taken for return to the initial volume ranged from 8 to 50 sec.

The volume changes consequent upon direct stimulation of the sympathetic efferent pathway have the same general form and time relations as the reflex response. The stellate ganglion and the lumbar sympathetic chains were stimulated by pinching or by break induction shocks. After a latent period the volume reaches a minimum rather more rapidly than the reflex response; the minimum was attained in an average time of 3·2 sec. from the beginning of the change (range 0·8–6 sec.) against 4·2 sec. (range 2·5–10 sec.). The differences between the reflex and direct latent periods will be discussed in the next section.

Central reflex time

The latency of the reflex response was invariably greater than the latency of the response to direct stimulation of the sympathetic chains. The error incurred by including the time of conduction along the afferent nerve was less than the error of the method; altering the length of nerve involved did not appreciably alter the latent period.

A survey of the observations showed that the shortest differences recorded were of the order of 0·4 sec. The method involved the estimation of the latent period before and after the exposure and section of the chains; although care was taken that the state of the vessels was apparently the same during corresponding observations, there was sufficient variation in the periphery to make many of the times much longer. It is clear that accurate estimates of the central reflex times require a method which will allow more rigid control of these factors.

Summation

Single break induced shocks were given to nerve trunks at intervals of 20 and 50 m./sec. Spatial summation of responses was indicated when subliminal and submaximal stimuli were applied to the same or to different sensory nerves. The same result obtained on stimulation of preganglionic and postganglionic fibres to the hind limb. The preganglionic fibres were stimulated in the lumbar chains, while the postganglionic were stimulated by applying the two electrodes to the distal halves of the cut sciatic nerve after splitting the nerve longitudinally.

At present it is not possible to discuss the significance of these findings. Spontaneous variations in the periphery precluded the construction of accurate

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curves relating summation of effects to stimulus interval. Further analysis is needed to define the site of summation and the factors underlying its production.

DISCUSSION

Using a sensitive optical recording method we have found a short-lived volume change in the paws of anaesthetized cats following many types of sensory stimulation. The records of the volume changes show the same spatial and temporal features as the records of digital volume changes in man. Stürup *et al.* [1935] find a latent period of 2–3 sec. in man, while in cats the duration is 0·5–2·0 sec.; other investigators have not recorded their findings, but from the published records the latent period is in all cases a matter of a second or more. Following the latent period there is a sharp decrease of volume, followed by a slower return. So strong are the resemblances between the records obtained from man and the cat that one is tempted to assume that identical physiological phenomena are being recorded in the two species.

The basis of the response is a vascular change. Skeletal muscle activity was precluded by the use of curarine, and the only means of producing such rapid changes of volume is by shift of fluid from the paws. Hallion and Conte deduced an underlying vasoconstriction from the accompanying change of shape of the arterial pulse tracing. Hertzman & Dillon [1939] and Burton [1939] emphasize the decrease of amplitude of the pulsations accompanying the reduction of volume, and it can be seen in the published tracings of other workers. Wilkins, Doupé & Newman [1938], Burton [1939], and Burton & Taylor [1939], have shown that there is a reduction of inflow of blood into the finger during the decrease of volume. More direct evidence is provided by Mulinos & Shulman [1939]; from direct observation of the capillaries of the nail bed they concluded that the reduction of flow depended upon some happening on the arterial side of the capillary. The persistence of the volume change when the main artery to the limb was occluded, found also by Bolton, Carmichael & Stürup [1936], and Lieb, Mulinos & Taylor [1936], indicates that the change of volume is not secondary to a reduction of blood flow above the level of the paw itself. Our evidence is that the greater part, if not the whole, of the volume change arises in the pads. We may deduce therefore that the volume change is dependent upon a change in skin vessels. The precise site of this change and the relative part played by the different vessels remains to be cleared up.

There can be no doubt that the vaso-constriction is of neurogenic origin. Not only does it occur with the limb circulation temporarily stopped but also in the acutely adrenalectomized animal. Furthermore, the latent period of the vaso-constriction caused by a constrictor drug (adrenaline) injected into the internal jugular vein was in our hands never less than 7·5 sec. Finally, interruption of the nervous pathway abolishes the response. In man, for example,

Stürup *et al.* [1935] and Peters [1939] could not obtain the response in the finger following avulsion of the brachial plexus, and we have found that the response is abolished by section of the sympathetic chain at the appropriate level.

The connector neurones leave the spinal cord with the anterior roots. Centrifugal stimulation of anterior roots in the lumbar region caused decrease of volume of the corresponding hind paw while centrifugal stimulation of the cut posterior roots did not. Section of the sympathetic supply to the limb also abolishes the response. We have found that removal of the stellate ganglion or the lumbar sympathetic chain prevented the change of paw volume although stimulation of the peripheral cut end of the nerve demonstrated that the vessels were still reactive. Stürup *et al.* [1935], Bolton *et al.* [1936], and Burton [1939] have found that cervical or lumbar ganglionectomy is followed by absence of vaso-constriction of the corresponding digits. The impulses then pass down fibres in the somatic nerves; we have found that stimulation of the peripheral end of the cut sciatic nerve produces a decrease of hind-paw volume. Malmejac & Haimovici [1936] showed that in dogs section of the nerves to the muscles and veins of the hind limb decreased the change of paw volume, and that subsequent denervation of the arterial walls abolished the small residual response. The experimental findings indicate that the impulses leave the cord, pass into the sympathetic chain and are relayed out to the paw or digit along fibres which run in the somatic nerves. This course represents the final common path of the reflex vaso-constriction in both man and cat. The work of Malmejac & Haimovici [1936] shows that a small part of the diminution of paw volume may be dependent upon the peri-arterial nerve net. Bearing in mind the conclusion of Gilding [1932] that this sympathetic plexus does not conduct impulses to the peripheral vessels, it seems probable that the small decrease of volume is secondary to a constriction of the main artery due to impulses conducted along this pathway. The majority of the conducting fibres, however, run in the somatic nerves. Applying further the conclusions of Gilding [1932] it seems that these fibres leave the main nerve trunk to reach the pads or digital skin in the local sensory nerves, but no direct confirmation of this is recorded.

Stimulation of cut posterior roots produced a response only when the central end was stimulated; no change in paw volume followed stimulation of the peripheral cut end. As we have pointed out, the absence of dilatation may have been due to an already maximal dilatation of the skin vessels. The anaesthetic itself may have played some part in this; de Waele, van de Velde & Braeye [1933] have shown that deepening of ether narcosis abolishes the classical effect.

The pathway through the central nervous system has not been traced. Bolton, Williams & Carmichael [1937] found that in two cases of spinal cord lesions there was no evidence of reflex activity through intact sympathetic ganglia alone but that a path in the cord is involved, while Bolton *et al.* [1936] showed that total transection of the cord by crushing in man prevented the appearance

of the reflex to deep breathing below the lesion level. We have not, however, been able to demonstrate a reflex pathway confined to the spinal cord. Certainly destruction of the cord abolishes the responses but so also does acute section in the cervical, dorsal, or lumbar regions in the anaesthetized animal. The cause of this may be, on the one hand, the acute upset of the cord. Sherrington [1906] showed in dogs that all reflexes, including vaso-pressor, were lost for some hours below the transection after withdrawal of the anaesthetic, the return of vascular reflexes requiring even some days. Although we used strychnine in an attempt to heighten any trace of the reflex, following the suggestion of Bayliss [1923], it is probable that we did not wait long enough to demonstrate it. On the other hand, the central path may be long; the long central reflex time indicates this, but the effect of the anaesthetic has not been determined. However long it may be, the path is complete in the brain stem below the level of the superior colliculi, shown by the persistence of the response after decerebration at this level. In man the cerebral cortex is not involved, typical responses occurring in spite of an extensive decortication [Williams & Scott, 1939] and diverse cerebral lesions [Stürup *et al.* 1935].

Reverting to the afferent side of the reflex arc, it is seen that the receptive field of the reflex is large and is not limited to the end-organs in any one area of the body. Impulses set up in this field converge on the centre to cause discharge of impulses along the final common path. It is noteworthy that the response is as easily elicited in man as in animals; sudden noises, stimulation of the skin and distending a balloon in the intestine are effective in both species. At present it is not possible to say whether any specific type of end-organ is involved. It is interesting to note also that the same methods of indirect stimulation produce marked vaso-constriction in the paw as produce dilatation of the pupil in the chloralosed cat.

The resemblances between the reflex in animals and man suggest that one and the same reflex is involved, and analysis shows that the anatomical plan is the same. Final discrimination of the site of action of the impulses is still needed. At what level this reflex path is complete within the brain stem is still undefined; certainly it is complete below the superior colliculi. Many more details of the anatomy require investigation, but the establishment of the reflex allows of its use as an index of changes in the central nervous system; for example, it will form an index of the arrival of impulses at the lateral horn cell or of changes of state of the cell itself.

SUMMARY

1. Reflex changes of paw volume of anaesthetized cats have been recorded by a sensitive optical method.

2. Various methods of sensory stimulation cause a transient decrease of paw volume. This is due to active constriction of the vessels of the pads.

3. The final common path of the reflex is represented by the sympathetic nerve supply of the limb. The central paths have not been traced but are complete below the level of the superior colliculi.

4. The latent period of the response is long; the central reflex time also appears to be long.

5. The reflex response can be used as an index of changes in the centres, and gives evidence of summation there.

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THE INFLUENCE OF POSTURE ON THE PULMONARY BLOOD VOLUME AND THE ALVEOLAR GAS TENSIONS

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In a recent series of papers Main has dealt with the problem of the fall in the tension of the carbon dioxide in the alveolar air which occurs on the assumption of the erect posture. In 1937 he suggested that it was due to an increase in pulmonary ventilation. Hitchcock & Ferguson [1938] disagreed with this and pointed out that the phenomenon took place without an increase in ventilation of the lungs. In two of the latter's subjects there was both a fall in the pulmonary ventilation and in the alveolar CO_2 tension on standing. In 1941 Main defended himself by applying a different interpretation to the term 'over-ventilation'. He pointed out that if the pulmonary ventilation was reduced, and at the same time the volume of blood circulating in the lungs was less, this latter volume could be over-ventilated with a consequent fall in CO_2 tension. That Main was, however, mainly concerned with ventilation and the factors affecting this, and not with circulatory phenomena, can be judged from the theme of his papers. In 1940, his opening sentence is: 'The cause of the overventilation in the erect posture is unknown', and he proceeds to investigate whether the fall in pressure within the carotid sinus which occurs on standing is responsible for the increase in breathing. He gives vasopressor drugs to see whether the pressure changes they produce will influence alveolar CO_2 tension by their effect on pulmonary ventilation. He concludes that the respiratory stimulant is a 'relative cerebral anaemia'. These views are similar to those expressed by Turner [1927], who states that the very moderate increases in respiratory activity which occur on standing can be very easily explained as the result of carotid sinus reflexes and diminished cerebral circulation.

The alteration in vital capacity which was pointed out by Hill [1895] has been brought forward as a factor influencing respiration. Bohr [1907] agrees that the vital capacity increases on standing, and Brunton [1908] points out that since the diaphragm has to raise and lower the viscera when one is recumbent, but merely to move them horizontally when upright, this is the key to the limitations which posture places on breathing. Christie & Beams [1922] found from the average of 290 normal subjects that the vital capacity decreases by

5·5% in the recumbent position. Hurtado & Fray [1933] found slight decreases in vital capacity in the supine position. Wilson [1927], on the other hand, observed no marked change in vital capacity in different postures.

Chest measurements were made by Livingstone [1928] who used radiological methods; he demonstrated that the capacity of the chest is least when supine. McMichael & McGibbon [1939], on the other hand, were unable to show any change in the size of the chest. That the capacity of the chest is not entirely dependent on the position of its parietes has been clearly demonstrated by Glaser & McMichael [1940]. They found in a series of normal subjects that the withdrawal of 380 c.c. of blood by venesection was accompanied by an increase in vital capacity averaging 153 c.c. That the circulating volume can encroach upon the pulmonary air space has been shown by Drinker, Peabody & Blumgart [1922]. This is very understandable when the passivity of the pulmonary circulation is considered, as pointed out by Drinker, Churchill & Ferry [1926], Hall [1925], and Brenner [1935].

The experiments described in this paper were carried out in order to examine this phenomenon and to seek an explanation.

THE APPARATUS

This consisted of a tilting table and equipment for the collection and analysis of alveolar samples.

The tilting table was built on a strong frame which was securely bracketed to the floor. It was so balanced that when a subject was lying on it he could be easily tilted into any required position by an assistant and maintained at that angle by means of a bracket and bolt.

The alveolar samples were collected by means of a Haldane-Priestley tube held in a frame. Evacuated sampling tubes were used. Only end-expiration samples were taken, and the results are expressed as a percentage of the prevailing atmosphere. The 'resting state' was reached by the subject before samples were delivered.

THE PROCEDURE

Three normal subjects were used, and upon these repeated observations were made in which agreement was reached. It was first established that these showed a fall in alveolar CO_2 tension when they were tilted into the erect posture. The results of one such experiment are given in Table 1.

The reverse procedure, that is, tilting the subject into the horizontal after standing, causes a rise in the alveolar CO_2 percentage (see Table 2).

Logically three processes might be causing the fall in the alveolar CO_2 tension when the subject becomes erect:

(1) The volume of blood exposed to the lung gases may be reduced.

(2) There may be an increase in pulmonary ventilation.

(3) A combination of the above two processes may be taking place.

TABLE 1

Posture	Time		Alveolar CO_2 %
	min.	sec.	
Supine	—	—	5·58
,,	—	—	5·57
Erect	0	30	5·43
,,	1	25	5·13
,,	2	45	4·92
,,	3	57	5·16
,,	5	35	5·03
,,	7	02	4·92
,,	8	53	5·05

TABLE 2

Posture	Time		Alveolar CO_2 %
	min.	sec.	
Erect	—	—	5·28
Supine	1	10	5·64
,,	2	35	5·74
,,	4	52	5·77
,,	7	14	5·72
,,	9	50	5·71

This paper is divided into three sections. The first two of the above processes are examined, and then the last section deals with other hypotheses that have been brought forward.

I. ALTERATIONS IN THE VOLUME OF BLOOD EXPOSED TO THE LUNG GASES IN CHANGE OF POSTURE

It was first decided to examine whether the gravitation of blood to the dependent parts played a role in the alteration of the tensions of the gases in the lungs. The procedure adopted was similar to that in the experiment in Table 1, only on this occasion the legs were firmly bandaged from the toes to the groin with 3 in. elastic bandages. The results of two of these experiments are given in Table 3.

TABLE 3

	Posture	Time		Alveolar CO_2 %	
		min.	sec.		
Exp. 1	Supine	—	—	5·44	
	,,	—	—	5·54	
	Erect	0	45	5·41	With bandages on the legs
	,,	2	00	5·28	
	,,	3	15	5·42	
	,,	4	45	5·28	
	,,	6	15	5·43	
	,,	8	00	5·29	
Exp. 2	Supine	—	—	5·51	With bandages on the legs
	Erect	2	15	5·47	
	,,	4	00	5·33	
	Supine	—	—	5·55	With bandages removed
	Erect	2	45	5·12	
	,,	4	00	5·09	

These results show that when the lower limbs are bandaged the fall of the alveolar CO_2 tension when the subject assumes the erect posture is now reduced.

In Exp. 2 in Table 3, the changes of posture with and without the bandages were all carried out on the one occasion.

Reversal of the order of the delivery of the samples while the legs were bandaged gave similar results. The average of such a series showed that on standing the alveolar CO_2 was 5·28% and only rose to 5·31% on lying down.

To establish firmly that the bandaging of the legs was responsible for preventing the fall in the alveolar CO_2 tension, the above experiments were repeated in a modified form. The legs were bandaged and samples were delivered in the supine position; the subject was then tilted into the vertical position and further samples were collected. The bandages were now rapidly removed by assistants and further lung samples were taken. The results of such an experiment are given in Table 4.

TABLE 4

Posture	Time		Alveolar CO_2 %
	min.	sec.	
Supine	—	—	5·32
„	—	—	5·36
Erect	1	00	
„	2	30	5·37 (av. of three determinations)
„	4	00	
Bandages removed	4	30	
Erect	5	30	
„	7	30	4·95 (av. of three determinations)
„	9	30	

The fall in tension of the alveolar CO_2 might be explained by the fact that when the subject stands a certain amount of the circulating volume gravitates into the dependent parts, where it becomes 'cut off' from the main 'effective' circulation and thus reduces the volume that freely circulates through the lungs. Let us neglect for the moment any changes that might occur in pulmonary ventilation and let us assume that this is not altered. In these circumstances the smaller circulating pulmonary volume of the upright posture will be relatively over-ventilated, with a consequent fall in the CO_2 tension. This will be reflected in a lower alveolar CO_2 tension. This hypothesis is supported by the above experiments. To confirm this a further series of tests was made, only this time pressure cuffs acting as venous tourniquets were employed.

Samples were delivered standing, following which the pressure cuffs were applied to the thighs at 100 mm. Hg, and then the subject was tilted into the horizontal. These cuffs should retain the blood volume of the lower limbs in position. Samples were now delivered in the supine position before and after the pressure on the cuffs was released. In Table 5 are given the results of one of these tests.

TABLE 5

Posture	Time		Alveolar CO_2 %
	min.	sec.	
Erect	3	00	5·19
,,	4	30	5·15
Cuffs pumped up to 100 mm. Hg			
Supine	8	30	5·06
,,	9	45	5·18
Cuffs released			
Supine	13	00	5·43
,,	15	00	5·55

It might be argued from this that when the subject lies down the tourniquets prevent a volume of blood in the lower limbs from flowing back into the main circulation. Thus the pulmonary circulation and alveolar CO_2 percentage remain unaffected. But both rise when the tourniquets are released.

It was shown that carbon dioxide accumulation in the isolated limbs was not the responsible factor, by occluding the *four* limbs with tourniquets above systolic pressure in the supine subject for similar periods of time and finding no significant change in alveolar CO_2 tension when the cuffs were released.

The relationship between vital capacity, alveolar CO_2 tension and posture

The vital capacity is considered under certain circumstances to be an expression of the volume of blood in the lungs. Thus a reduction in vital capacity might be due to an encroachment on the air volume of the lungs by an increase in the pulmonary circulating volume. Therefore an examination of the relationship between vital capacity, posture, and the tension of the lung gases falls appropriately into this section.

The following experiments were designed to demonstrate that under those circumstances where the vital capacity was reduced by allowing a volume of blood to flow towards the thorax there was at the same time a change in the tension of the alveolar CO_2.

TABLE 6

Posture	Alveolar CO_2 %	Vital capacity c.c.
Supine	5·39	3·833 (av. of six determinations)
Tourniquets applied to thighs at 100 mg. Hg		
Legs lifted to 40° by assistant	5·33	3·830 (av. of five determinations)
Tourniquets released		
Legs lifted to 40° by assistant	5·56	3·390 (av. of five determinations)

The above experiment had to be carried out in two stages, as the simultaneous delivery of alveolar and vital capacity samples is not practicable. These results suggest that the blood from the raised limbs flows into the thorax when the tourniquets are released, and causes a simultaneous fall in vital capacity and a rise in CO_2 tension. The results in Table 7 lend support

to this hypothesis. In this instance the subject was tilted into a head-downward position at an angle of 25°. Tourniquets had been applied to the thighs. The release of these tourniquets allowed the dammed blood to flow towards the thorax, causing the vital capacity to fall and the alveolar CO_2 tension to rise.

TABLE 7

Time		Vital capacity	Alveolar CO_2 %
min.	sec.	c.c.	
		Subject supine and horizontal	
8	10	3·900 ⎫	
9	50	3·900 ⎪	5·67
10	40	4·000 ⎬	
11	45	3·800 ⎭	
12	45	Tourniquets applied to lower limbs at 100 mm. Hg	
14	15	4·000 ⎫	5·61
15	30	4·100 ⎭	
16	05	Tilted head down at 25°	
16	45	4·100 ⎫	
17	50	3·900 ⎬	5·57
18	35	4·050 ⎭	
19	00	Tourniquets released	
19	30	3·650	5·93
20	40	3·750	5·78
21	15	3·800	5·80
22	38	3·850	5·85
23	10	3·750	5·85

The vital capacity and alveolar CO_2 measurements were taken separately by repeating the tilting procedure and application of the tourniquets and adhering as closely as possible to the same time intervals. There is no doubt in the mind of the subject about what is happening when the tourniquets are released. There is a marked sense of suffocation as the blood wells into the upper parts of the body.

Tilting down (head first) without the application of tourniquets causes a reduction in vital capacity. The results on one subject show that when he is horizontal the vital capacity = 3·880 c.c. (average of five determinations) and when tilted vital capacity = 3·675 c.c. (average of four determinations). Many other methods can be devised to show the relationship that exists between vital capacity and the distribution of the circulating volume. A simple procedure was to apply the elastic bandages to the legs of a horizontal subject. His vital capacity was then found to be 3·800 c.c. When the bandages were removed, it increased to 4·100 c.c.

II. ALTERATIONS IN THE PULMONARY VENTILATION WITH CHANGE OF POSTURE AND ITS INFLUENCE ON THE TENSION OF THE ALVEOLAR CARBON DIOXIDE

The next question to be examined was, to what extent are the changes in gas tension due to alterations in pulmonary ventilation?

TABLE 8

Posture	Minute volume litres	Alveolar CO_2 %
Lying	5·28	5·50
Standing	5·64	5·01
Standing with legs bandaged	5·56	5·32

Examination of the results in Table 8 shows that for this subject there is an increase in pulmonary ventilation on changing from the lying to the standing position. Is this rise in minute pulmonary ventilation from 5·28 to 5·64 l. responsible for the fall in alveolar CO_2% from 5·50 to 5·01? Voluntary increases in pulmonary ventilation while the subject maintained a constant posture were carried out and at the same time alveolar samples were collected. This experiment was carried out while the subject was seated. A bivalve mouthpiece was used and the minute volume was recorded on a dry spirometer. At a certain point in his respiratory cycle the mouthpiece was switched over to a Haldane-Priestley alveolar sampling tube and an end-expiration sample was collected. This was carried out by means of a sliding mouthpiece as used by Mackay [1939]. When the results were plotted it was found that for a change in alveolar CO_2 tension of 5·50–5·01% an increase of 2·7 l./min. in pulmonary ventilation was required, as compared with 0·36 l. which is the increase in the postural change. It is reasonable to conclude from these figures that the part played by pulmonary ventilation in affecting the tension of the lung gases in postural changes is a minor one. This supports Hitchcock & Ferguson [1938], who showed that in some subjects there might be no alteration—or even a reduction—in pulmonary ventilation on changing from the horizontal to the vertical position, and yet a fall in alveolar CO_2% will occur.

Further evidence against changes in ventilation playing a dominant part is given in Table 9. In this instance a change in posture from standing to stooping caused a rise in the CO_2%, but it was accompanied by no significant change in pulmonary ventilation.

TABLE 9

Posture	Minute pulmonary ventilation	Alveolar CO_2 %
Supine	5·30	5·30
Standing	6·00	4·80
Stooping	5·99	5·58

This stooping phenomenon was reported by Turner [1927] who quoted it in support of his hypothesis of cerebral ischaemia. He held that when a subject stoops the cerebral ischaemia is relieved and a respiratory stimulant is removed, with a consequent depression of breathing and a rise in CO_2 tension. It is difficult to understand what is the mechanism involved in this.

In the example in Table 10, when the subject stoops the alveolar CO_2 tension rises, there is then a tendency after a period for it to approach its original level.

TABLE 10

Posture	Alveolar CO_2 %
Standing	4·96
"	5·00
Stooping	
1·00	5·67
2·45	5·51
5·45	5·59
7·50	5·59
9·45	5·31

Two possible explanations seemed to present themselves. First, the lowering of the thorax into the horizontal position might increase the volume of the pulmonary pool; secondly, the compression of the abdominal contents might thrust a further volume of blood into the lungs. The first point has already been examined for the subject in the supine posture. To investigate the second the following procedure was instituted. The subject first delivered alveolar samples standing. Pressure cuffs were applied to the thighs and the subject then lay in the supine posture. Another sample was delivered. The legs were now raised on a board by an assistant until they were at right angles to the trunk. Because of the tourniquets no blood should have flowed from the lower limbs, yet this manœuvre caused an increase in alveolar CO_2 tension. An example of such an experiment is given in Table 11.

TABLE 11

Posture	Alveolar CO_2 %	Time min.	Time sec.
Standing	5·34	0	00
	5·31	1	10
Tourniquets applied to thighs at 100 mm. Hg	—	1	30
Supine	5·34	3	55
	5·40	4	45
Legs placed at right angles	—	5	10
	5·71	5	48
	5·72	6	30

To what extent is the placing of the limbs at right angles to the trunk when it is supine comparable to the stooping posture when the trunk is in the prone position? The vital capacity measurements taken while the subject is supine show a reduction when the limbs are lifted into the right-angle position, although venous tourniquets are applied. This supports the suggestion that this procedure forces blood from the abdomen to the thorax. On the other hand, there is no significant alteration in the vital capacity when a subject changes from standing to stooping, so that we are unable to produce evidence of an increase in the pulmonary blood pool by this method. Should the volume of blood increase in the lungs when the subject stoops it is not shown by vital capacity measurements (see Table 12). This stooping phenomenon still remains unsolved.

The possibility of changes both in pulmonary circulating volume and ventilation occurring at the same time must be admitted. In the subjects of these experiments changes in ventilation played a minor part.

III. THE EXAMINATION OF OTHER HYPOTHESES

The relationship between lung volumes, posture and alveolar carbon dioxide tension

Attention was now turned to observe whether the alteration in lung volumes (e.g. residual air volume) that occurs with changes of posture has any effect on the tensions of the lung gases.

Hitchcock & Ferguson [1938] advanced the theory that the fall in alveolar CO_2 tension on standing is due to the simple dilution resulting from the increased volume of functional residual air (supplemental air). Against this hypothesis is the possibility that should the volume of the air present in the lungs increase, the lung tissues may not be as well ventilated. This should happen when the residual air increases and when there is no change in the minute volume.

In order to examine this hypothesis experimentally an attempt was made to alter the functional residual air and at the same time maintain a constant posture. This was done by tightly strapping the chest with the subject in the sitting position. After samples were collected the strapping was removed and the sampling repeated.

TABLE 12

	Functional residual air c.c.	Alveolar CO_2 %	
Subject in the sitting position with chest strapped	467	5·60} 5·55}	5·58
After the release of the strapping	917	5·45} 5·43}	5·44

These results show a slight fall when the residual air increases.

Cotton [1939], Mackay [1940, 1942] and Marenzi & Costoya [1941] have reported that in some subjects a shallow alveolar sample may give a higher CO_2 tension than a deeper one. Where the functional residual air is altered there will be an alteration in the depth of the expiration when an alveolar sample is delivered. In the subject in Table 13 this expiration (end-expiration) varied from 1·605 to 300 c.c. It must, however, be remembered that the above workers carried out their experiments under conditions which ensured a constant residual air lung volume, and their samples were taken from expirations of ranging depths so that their methods are not really comparable.

In Table 13 are figures showing the changes that occur in some of the lung volumes in different postures. Those which show the most marked changes are

the supplemental air volumes, but there is no relationship between changes here and the alveolar CO_2%. The only section in which the variations in the lung volumes etc. and the alveolar CO_2 run parallel is that of the minute volume.

TABLE 13

Posture	Vital capacity c.c.	Residual air (supplemental air) c.c.	Tidal volume c.c.	Minute volume c.c.	Alveolar CO_2 %
Standing	4200	1283	685	6500	5·02
Stooping	4180	1605	813	6910	5·76
Supine with legs at right angles, venous tourniquets on thighs	3550	300	694	5620	5·54
Lying	3900	314	613	5440	5·45

The relaxed position

Higgins [1914] has pointed out that the proprioceptive stimuli increase in standing, and suggests that this has an effect on the tension of the gases in the lungs. This was investigated in the following manner. Two strong pulleys were fixed to the side of the tilting table. A board upon which the feet could rest was attached by strong ropes, passing over the pulleys, to a heavy weight. With the shoulders fixed in position by means of a large towel, which was fastened to the table, the weight was lifted from the floor by pressing on the board with the feet. This brought into action the muscles that are used in standing. While these muscles were in action alveolar samples were taken. The average of four experiments showed that while relaxed the alveolar CO_2% was 5·51, and after pressing on the weight for 4 min. was 5·55.

Blood-pressure effects

Main [1940] has investigated the effect of raising the blood pressure on respiration. He has pointed out that when a subject stands there is a drop in the pressure in the carotid sinus; this should act as a respiratory stimulant. Main administered drugs and produced a change of over 20 mm. Hg in the blood pressure without any effect on the tension of the alveolar carbon dioxide. Blood-pressure readings recorded on one of the subjects of the above experiments when tilted into the upright position showed a smaller variation than those of Main. It is reasonable to assume that this small fall in blood pressure is playing a stimulating part, despite the inhibitory effect of CO_2 removal from the arterial pulmonary air which occurs when a subject stands. This subject did not show a reduction in pulmonary ventilation but a small increase, which must have been due to the only respiratory stimulant working, that is, a fall in blood pressure in the carotid sinus.

Summary

1. When a subject adopts the erect posture a fall in the tension of the alveolar carbon dioxide takes place.

2. Experiments were performed the results of which justify the conclusion that the main factor responsible in these subjects for this fall is a reduction in the volume of blood to which the lung gases are exposed.

3. The part played by an increase in pulmonary ventilation is a minor one.

4. Various hypotheses previously brought forward are examined.

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CHRONIC SUPRARENAL DEFICIENCY AND ITS EFFECTS ON THE RESPONSES OF THE ISOLATED INTESTINE IN THE RABBIT

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Disturbances of carbohydrate metabolism and supersensitivity to various drugs are among the prominent signs exhibited by suprarenalectomized animals. Little, however, is known about the way in which these abnormalities are caused, and it is uncertain whether they are localized in particular organs or are manifestations of a general cellular deficiency. Work on tissue slices has been carried out and a reduced oxygen uptake observed in the kidney [Crismon & Field, 1940; Russell & Wilhelmi, 1941]; a similar decrease found in minced brain, however [Himwich, Fazikas, Barker & Hurlburt, 1934], was associated with any type of hypotension, whether of suprarenal origin or not. The phenomenon may, therefore, not be caused by lack of cortical hormone.

Except for the frequently investigated frog's muscle, little work has been done on the isolated organs of animals suffering from suprarenal deficiency, and it is not well understood which functions of the body are the first to be affected and which pathological signs are secondary phenomena. That glycogen storage in the liver is subnormal is firmly established; it is, however, not known whether the low blood sugar, the serious consequences of fasting. the poor conversion of endogenous protein into carbohydrate [Wells & Kendall, 1940; Long, Katzin & Fry, 1940], the incomplete utilization of lactic and pyruvic acid [Britton & Silvette, 1934; Buell, Anderson & Strauss, 1936; Lewis, Kuhlman, Delbue, Koepf & Thorn, 1940], and the increased utilization of glucose [Seckel, 1940; Evans, 1941; Ingle, 1941] are due mainly to dysfunction of the liver or equally to abnormal metabolic processes in other tissues. Though a deficient carbohydrate metabolism was detected in liver slices [Koepf, Horn, Gemmill & Thorn, 1941], lactate formation in striated muscle was also found abnormal [Buell, Strauss & Andrus, 1932; Ochoa, 1933]; the important role played by the liver in the signs of suprarenal deficiency might, therefore, simply be a consequence of its central position in metabolism

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rather than a result of a primary damage not suffered by other organs. The rabbit's intestine seemed to be a preparation in which both disturbances of carbohydrate metabolism and abnormal responses to drugs should be easily detected, provided these changes were ubiquitous and therefore included the intestinal musculature or its nervous elements. The utilization of carbohydrate can easily be followed on isolated pieces of rabbit's intestine on account of their regular rhythmic activity which is only kept up in the presence of an adequate supply of carbohydrate; the responses to drugs are manifold, as some substances affect mainly the longitudinal and some mainly the circular coat, and as stimulation or inhibition may be caused by action on the muscle, on Auerbach's plexus, or on both.

Experiments were, therefore, carried out on strips of small intestine obtained from rabbits showing signs of severe suprarenal deficiency. Examination was made of their responses to carbohydrate and related substances, to poisons affecting carbohydrate metabolism, and to drugs which cause stimulation or inhibition of movement and tone. The results were compared with those on normal animals or on fasting controls not deprived of their suprarenals.

OPERATION AND SURVIVAL TIME

After a preoperative injection of 4 mg. atropine sulphate, suprarenalectomy was performed under ether anaesthesia and with aseptic precautions. The ventral route was chosen and the glands exposed by a long abdominal incision in the midline. After separating the gland as far as possible from the surrounding tissues, it was seized with a fine pair of trachoma forceps which could be fixed in the closed position by means of a screw. A ligature was tied beyond the forceps and the gland excised.

Great discrepancies are found in the literature concerning the survival time of suprarenalectomized rabbits. Whereas some authors [for instance Rothschild, 1915; and Girndt, 1925] report death within the first 24 hr., other workers were able to keep the animals alive for longer periods. Hultgren & Andersson [1899] were the first to apply aseptic precautions and to describe survival times of 5–15 days and a small percentage of complete recoveries. Similar figures are found in more recent work [e.g. Cope, Capnick, Lambert, Pratt & Verlot, 1939], whereas other experimenters state that indefinite survival is the rule [Baumann & Holly, 1923]. In the material used for the present investigations (61 rabbits), 30% died within 24 hr. of the operation, 20% survived indefinitely, and 50% developed chronic insufficiency with an average survival time of 10 days. The frequency distribution of the survival times in this last group is represented in Table 1.

TABLE 1. Survival times of suprarenalectomized rabbits developing chronic deficiency

No. of days	1	2	4	5	6	7	8	9	10	11	12	15	19	37	59
No. of rabbits	5	1	3	3	3	1	3	1	3	?	1	1	1	1	1

The first twelve suprarenalectomies were done in one, all the subsequent operations in two stages; no essential change in the frequency of early deaths or length of survival times, however, resulted from this change of procedure. In the animals operated in two stages, the more difficult excision of the right gland was always done first; nevertheless, recovery from the first operation was always uneventful; the result of the second operation, however, was completely unpredictable. Treatment was confined to the day of operation, and consisted of subcutaneous injections of cortical extract ('Eucortone', Allen and Hanburys, 1 ml. per dose) given immediately before, immediately after, and at one- or two-hourly intervals following the suprarenalectomy. Intravenous glucose-saline injections and treatment with sympatol and pholedrine were also tried. Saline instead of drinking water was given for the first night. Immediate recovery from the operation was always rapid, and the rabbit was usually sitting up within 15 min. of cessation of anaesthesia. In 30% of the animals, however, this recovery was followed, after a latent period of 3–5 hr., by muscular weakness, poor circulation, occasional diarrhoea, and death. No therapy whatsoever caused any but a very transient improvement. The cause of death was evidently the deteriorating circulation which appeared as refractory to treatment as it is in surgical shock. Cortical extract, at least in the amounts available (up to 6 or 7 ml.), was apparently completely inefficacious. Very young rabbits more frequently developed this acute circulatory failure than older animals.

Another group of rabbits, amounting to about 20%, showed some loss in body weight during a period of about 7–10 days after the operation; later on, their weight again rose and the animals gradually became indistinguishable from normal rabbits. Though accessory or regenerated cortical tissue was found on macroscopic inspection in most (80%) of these animals, it could not be found in all cases; histological examination was, however, not carried out.

The third group of rabbits is the only one with which this paper is concerned; it comprises those animals which develop a chronic suprarenal deficiency, which may prove fatal at any time between the 2nd and the 59th postoperative day, most animals, however, succumbing between the 7th and 12th day. The question, of course, arises, whether these rabbits survived because the operation was not as complete as in the first group, in which death occurred within a few hours of the suprarenalectomy. In spite of careful search (restricted, however, to macroscopic examination), traces of suprarenal tissue were only found in four of the chronically deficient animals; it is, therefore, probably correct to assume that this state develops either in the complete absence or, occasionally, in the presence of minute amounts of suprarenal cortex.

SIGNS OF CHRONIC SUPRARENAL DEFICIENCY IN THE RABBIT[1]

As early as 1899, Hultgren & Andersson described a fall in body temperature and body weight, muscular weakness and convulsions, as signs of suprarenal deficiency in the rabbit. In the present series of experiments, the first three signs were always present; convulsions, however, which are evidently of hypoglycaemic origin, occurred only in some animals. In addition, diarrhoea was frequently observed, and in those animals which survived for a sufficiently long period, an atrophy of the gastrointestinal tract and liver developed and produced a striking and characteristic picture. The weight of the viscera was considerably reduced: in normal animals of comparable size, liver, spleen and gastrointestinal tract (without contents) are responsible for 15·9% of the body weight, whereas the corresponding figure in suprarenal deficiency was 10·3%; details are summarized in Table 2. The internal and external diameters

TABLE 2. Weight of gastrointestinal tract, spleen and liver as percentage of body weight

	Normal rabbits			Suprarenalectomized rabbits		
No.	Weight of viscera, % body weight	Body weight kg.	No.	Weight of viscera, % body weight	Body weight when killed kg.	Days after completion of suprarenalectomy
1	19·0	0·80	80	11·0	1·0	10
2	16·5	0·88	76	13·0	1·11	11
3	15·6	0·93	78	11·6	1·24	8
4	14·5	1·16	82	10·3	1·28	6
5	14·0	1·65	84	11·0	1·27	5
Mean	15·9		65	7·6	1·57	37
			83	7·7	1·74	6
			Mean	10·3		

of the intestine, even in the relaxed condition, were smaller than usual, and, in addition, a tendency of the intestine to spastic contraction of its circular coat gave it a string-like appearance absent in the normal animal. Mechanical stimuli, as for instance the attempt at tying the intestine over the glass tubing leading to the volume recorder, caused prolonged violent constriction of the muscle in most of the deficient rabbits. The same phenomenon was observed by Fowler & Cleghorn [1942] in the cat. Control observations on fasting rabbits did not reveal similar changes, neither was the fall in body weight of normal animals during fasting as rapid as that of suprarenalectomized rabbits, in spite of the fact that, until immediately before death, the operated animals took at least some food. Most animals refused dry food after the operation, but frequently ate greens as long as they were able to sit up. As the deficiency increased, some rabbits drank large quantities of water.

The body weight was found to be the best measure of the clinical condition after suprarenalectomy. It fell steadily for a period of several days in all animals which survived the first 24 hr., and it kept on falling until death occurred in those rabbits which did not recover from the operation. The loss

in body weight suffered when the animals became moribund amounted to an average of 20% with a range of 11–32%.

The fall in temperature set in 3 to 1 days before death, and was much more variable in its progress. The lowest temperatures were seen in animals exhibiting convulsions; figures from 27 to 29° C. were then the rule. In other instances, death occurred, preceded by muscular paralysis, when the temperature was still 38° C.

In spite of careful observation, the condition of an animal was often difficult to assess. A rabbit which had appeared quite strong on the previous evening would sometimes die unexpectedly overnight, whereas, on other occasions, the falling body temperature and muscular weakness would give a warning of the state of the animal 2 or 3 days before it actually succumbed.

RESPONSES OF THE ISOLATED INTESTINE

Methods

A few preliminary tests on suprarenalectomized rabbits which did not exhibit any clinical signs had shown that the intestinal responses were normal in every respect. The animals were, therefore, not used until the deficiency had become conspicuous and the rabbit was nearly moribund. As death, however, frequently occurs quite suddenly and unexpectedly in these animals, occasional losses of rabbits were inevitable.

For the experiment, the rabbit was killed by a blow on the neck, the jejunum excised and transferred into glucose-free Tyrode's solution. The pieces which could not be used immediately were kept at room temperature. Pairs of strips (length 4–6 cm.) were suspended in a double organ bath in oxygenated, warm, but glucose-free Tyrode's solution, and the movements of the longitudinal muscle registered simultaneously with the intestinal volume, the latter providing a record of the activity of the circular muscle. Details of the method are published in a previous paper [Vogt, 1943]. Glucose was not added to the Tyrode's solution except where its own action was being investigated, because the rate of exhaustion of the intestinal muscle deprived of exogenous carbohydrate, and the greater clarity of the reactions of the circular coat, after carbohydrate lack had reduced or abolished the spontaneous pendulum movements, provided valuable information unobtainable in the presence of glucose. Examples of the usefulness of that method are found in some work on the normal intestine [Feldberg & Solandt, 1942; Vogt, 1943].

RESULTS

Although the circular muscle takes some part in the pendulum movements, its share is small compared with that of the longitudinal coat. The large amount of energy spent in mechanical work by the longitudinal coat is

probably one of the reasons why its spontaneous and induced activity disappear simultaneously if no carbohydrate is supplied with the suspension fluid; under the same conditions, on the other hand, the circular coat is not less, but frequently even more susceptible to stimulation by drugs, and often develops irregular outbursts of spontaneous contractions of considerable size. It follows from these properties of the two intestinal coats that the carbohydrate metabolism is best studied on the longitudinal muscle which requires exogenous energy for prolonged function, whereas the action of most drugs is better investigated on the circular coat. Exceptions are substances with an inhibitory action which can obviously best be seen on the longitudinal coat with its high spontaneous activity; acetylcholine, which depends particularly on glucose for its efficacy [Feldberg & Solandt, 1942], and histamine which has not much effect on the circular muscle.

Responses of the longitudinal coat

Exhaustion time. If a piece of normal intestine is suspended in glucose-free Tyrode's solution, a period varying from 30 min. to several hours elapses before the pendulum movements stop, or are, at least, very much reduced in size. This 'exhaustion time' is shortest in very young animals; 2 days' fasting, however, does not reduce it to any considerable degree. Amongst thirteen suprarenalectomized and severely deficient rabbits exhibiting some abnormality of their intestinal responses, there were seven in which exhaustion was rapid; in three the time required was still within normal limits, in four it was below the normal range and amounted to 10–20 min. In the gut of deficient rabbits, the energy stores available for muscular activity are thus occasionally found to be low, but more frequently they lie within the normal range.

Glucose. Normal intestine, suspended for an hour or two in glucose-free Tyrode's solution, loses its tone and its pendulum movements decrease. Glucose restores both (examples are reproduced in Feldberg & Solandt, 1942, Fig. 6) after a latent period, which increases with the period of deprivation of glucose. This effect was tested on the intestine of all the suprarenalectomized rabbits, and it was present every time. The example reproduced in Fig. 1 is typical and indistinguishable from the normal effect. The circular coat is not affected; the change in volume is probably passive.

Another effect of the administration of glucose to a piece of gut which has been deprived of its carbohydrate reserves is a restoration of the full response to acetylcholine [Feldberg & Solandt, 1942, Fig. 8]. The same result was obtained in the suprarenalectomized preparation.

The response of the longitudinal muscle to large doses of lactate was observed by Feldberg & Solandt [1942, p. 156] to disappear if glucose was withheld from the preparation. This effect and its restitution by glucose was

also those produced by other stimulating substances. Muscarine, potassium chloride and nicotine, for instance, were tested and found to elicit a diminished response in the 'exhausted' intestine; if glucose was then supplied, and the increase in tone caused by the sugar was allowed to pass off, the same drugs produced larger contractions, even if the spontaneous activity of the gut was no greater than before the treatment with glucose. Hence carbohydrate is probably indispensable for the response of the longitudinal coat to any stimulating drug. Here again, no difference could be detected between intestine of normal and suprarenalectomized animal.

Not only, therefore, is glucose utilized in a normal way in the intestine of the deficient rabbit for its spontaneous muscular activity, but it also supplies the energy for contractions elicited by drugs.

Pyruvate. The normal utilization of glucose reported in the foregoing experiments is in good agreement with the observation made by a number of authors of an increased utilization of glucose in the suprarenalectomized animal; the defective pyruvate metabolism observed by various workers (see p. 239) suggested that the same might not hold for pyruvate.

The result, however, did not differ from that obtained with glucose. In a piece of intestine, exhausted by prolonged suspension in a carbohydrate-free medium, pyruvate caused increase in tone and amplitude of the movements of the longitudinal coat. There was no indication that the response was less powerful than usual, and it was seen in all rabbits without exception. The circular coat was either not affected (Fig. 2a) or slightly stimulated (Fig. 2b). Thus the intestine of the suprarenalectomized rabbit utilizes in a normal manner both glucose and its cleavage product, pyruvic acid.

Phloridzin and iodoacetate. That the normal isolated intestine is capable of obtaining energy required for activity from non-carbohydrate, and, therefore, probably from protein, was demonstrated by Prasad [1935]. Experiments on diabetic or phloridzin-poisoned animals have further shown that the conversion of endogenous protein into carbohydrate is impaired by suprarenalectomy; if the intestinal musculature had a share in this deficient conversion, it might be expected that the gut which was taken from a suprarenalectomized animal would be more severely affected by poisons which prevent carbohydrate utilization than the intestine obtained from a normal animal.

Phloridzin and iodoacetate were chosen as inhibitors of glycolysis. The effect of phloridzin, in doses of 40 mg. added to a 75 ml. bath, was tested on the intestine of seven suprarenalectomized rabbits. The drug was given a few minutes after the gut had been suspended in glucose-free Tyrode's solution; at this time the pendulum movements were still vigorous, and the effect on the normal specimen resembled closely that illustrated by Feldberg & Solandt [1942, Fig. 17] in the presence of glucose. Of the seven deficient rabbits, two were more sensitive to phloridzin than normal controls: the pendulum move-

ments were not only decreased in size but ceased almost entirely for the period of exposure to the drug; this effect is shown in Fig. 3. In the other five rabbits, the inhibition of tone and rhythm was the same as that observed in normal animals.

In order to poison the gut with iodoacetate, a freshly prepared solution of the acid was neutralized and added to the bath so that the concentration was

Fig. 3. Fig. 4.
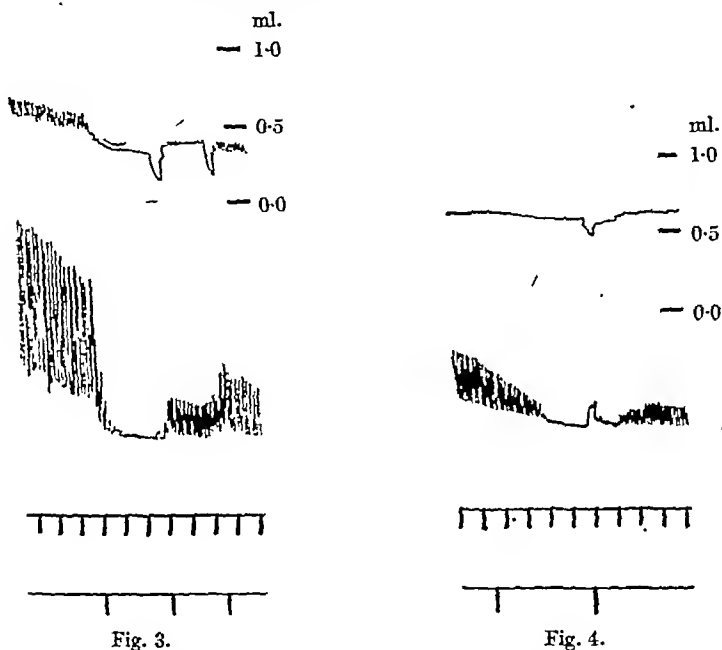
Fig. 3. Rabbit 64. Killed 11 days after extirpation of second suprarenal. Muscular weakness, low body temperature, atrophy of viscera. Strip of intestine suspended in glucose-free Tyrode's solution; record starts 4 min. later. Upper tracing: intestinal volume (Krogh chamber). Lower tracing: longitudinal muscle. Time: 30 sec. 40 mg. phloridzin are added at the first, and the strip is washed out at the second and third signal.

Fig. 4. Rabbit 64. For history see legend of Fig. 3. Piece of jejunum. Record starts 6 min. after the suspension of the strip in glucose-free Tyrode's solution. Upper tracing: intestinal volume (Krogh chamber). Lower tracing: longitudinal coat. Time: 30 sec. At the first signal, 10 mg. neutralized iodoacetic acid are added to the 75 ml. bath; they are washed out at the second signal.

1 in 10,000 or 1 in 7500 of the free acid. The experiment was always done on a recently suspended strip which was still performing large pendulum movements. After administration of iodoacetic acid, the excursions of the normal intestine's longitudinal coat very gradually decrease till the activity nearly or completely stops. The time required for that arrest to take place is usually between 7 and 10 min. These observations confirm the results of Feldberg [1943] but not those of Prasad [1935], who failed to find any action of iodoacetate in the presence of oxygen. In ten rabbits suffering from supra-

renal deficiency, the time required to arrest the pendulum movements was measured. It was found normal in nine and accelerated in one instance (Fig. 4); here inhibition of activity was complete within a little over 1 min. The rabbit in which this hypersensitivity was observed was one of those which had shown an exaggerated response to phloridzin. It was exceptional,

Fig. 5.   Fig. 6.

Fig. 5. Rabbit 64. For history see legend of Fig. 3. Strip of intestine suspended for 5 min. in glucose-free Tyrode's solution. The gut is still in the relaxing phase. Upper record: longitudinal muscle. Lower record: circular coat (Krogh chamber). Time: 30 sec. 1 mg. histamine acid phosphate is added to the 75 ml. bath at the first arrow, and washed out at the second arrow.
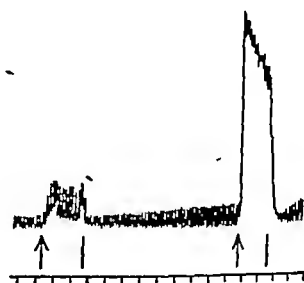
Fig. 6. Rabbit 20. Killed 9 days after extirpation of second suprarenal, when low body temperature and muscular weakness are shown. Piece of intestine suspended for 34 min. in glucose-free Tyrode's solution; amplitude of longitudinal muscle decreased to one quarter of its original size. Upper record: intestinal volume (piston recorder). Lower record: longitudinal coat. Time: 30 sec. At the first arrow, $1\mu g$. acetylcholine, and at the second arrow, $10\mu g$. acetylcholine are added to the 75 ml. bath. At the strokes, the drug is washed away.

therefore, to find a greater susceptibility to drugs which inhibit glycolysis in the deficient than in the normal intestine. Accordingly, the experiments provide no proof that suprarenal deficiency interferes with the conversion by the intestine of endogenous protein into carbohydrate.

Histamine. The toxicity of histamine is known to be much higher in suprarenalectomized than in normal animals, and this fact has even been suggested as the basis of a test for cortical activity, since resistance to histamine is restored by cortical extracts [Perla & Marmorston-Gottesmann. 1931]. Conse-

quently, it was of interest to know whether an increased susceptibility of all tissues, including intestinal muscle, accounted for the greater toxicity. In-
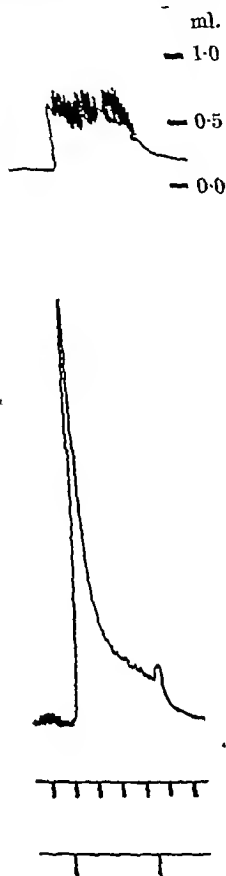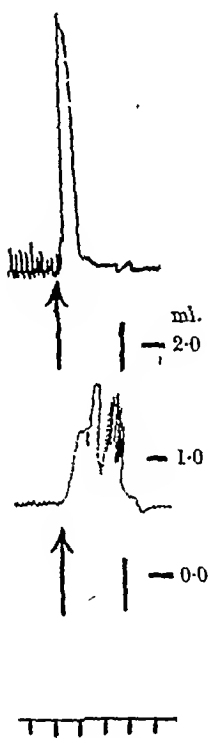
Fig. 7.   Fig. 8.

Fig. 7. Rabbit 34. Died 2 months after extirpation of second suprarenal, having exhibited ataxia and severe fall in body temperature. Intestine suspended 5 min. after death in glucose-free Tyrode's solution. Record starts 2 hr. later: amplitude of pendulum movements 1/10 of original size. Upper record: intestinal volume (Krogh chamber). Lower record: longitudinal muscle. Time: 30 sec. At the first signal, 0·8 mg. of a sample of natural muscarine is added to the 75 ml. bath, and washed away at the second signal.

Fig. 8. Rabbit 83. For history see legend of Fig. 2b. Strip of gut kept for half an hour at room temperature, then suspended for 1½ hr. in warm, oxygenated, glucose-free Tyrode's solution. During that period considerable decrease in the size of the pendulum movements. Upper record: longitudinal muscle. Lower record: intestinal volume (Krogh chamber). Time: 30 sec. At the arrows, 60 mg. KCl are added to the 75 ml. bath; they are washed away at the strokes.

testinal strips of eight suprarenalectomized rabbits were treated with varying doses of histamine. The usual increase in tone was observed, but never did

the responses exceed those of normal controls. Fig. 5 is an example, the intestine having been taken from the same rabbit which gave the abnormal reactions reproduced in Figs. 3 and 4.

Acetylcholine. It was discussed earlier on p. 244 how the response to acetylcholine depends, in the normal as well as in the deficient animal, on the period during which the isolated strip has been deprived of exogenous glucose. In this paragraph we are only concerned with the question whether the type of response differs from the normal reaction in the suprarenalectomized animal. Fig. 6 shows the response in a deficient rabbit, the strip having been partly exhausted by half an hour's work in the absence of glucose. 1 µg. acetylcholine causes mainly an increase in the excursions of the longitudinal muscle, whilst 10 µg. produce a large rise in tone which is moderately well sustained during the period of contact with the drug. The effect on the circular coat is negligible. These responses are neither qualitatively nor quantitatively different from those of normal tissue.

Other drugs. As already stated, the substances which are powerful stimulants of the circular coat were usually tested after prolonged suspension of the gut in glucose-free Tyrode's solution. Their action on the longitudinal muscle was observed at the same time, found normal in every respect, and will, therefore, not be described in detail. The drugs used were eserine, hypertonic sodium chloride, nicotine, muscarine and potassium chloride. Responses to the last two compounds are illustrated in Figs. 7 and 8.

Adrenaline and ephedrine. As both these substances produce relaxation of the longitudinal coat and inhibition of its rhythm, their action was tested on freshly suspended, still fully active, strips. The range of sensitivity to adrenaline is large in the normal intestine; conspicuous inhibition is, however, usually obtained with a solution of 1 in 10^8. Strips from twelve suprarenalectomized rabbits were examined, and the response to adrenaline was found within the normal range in every case. These results do not confirm Fowler & Cleghorn's [1942] finding in cats, according to which injections of adrenaline failed to cause relaxation of the bowels in animals suffering from suprarenal deficiency. Differences in the species or in the experimental conditions may account for this discrepancy.

Ephedrine has only a small relaxing effect on the normal isolated intestine. The responses of the gut taken from suprarenalectomized rabbits appeared neither larger nor smaller.

Responses of the circular coat

The mere inspection of the circular coat discloses abnormalities in certain suprarenalectomized rabbits. The tendency towards spastic constriction, especially when touched, was mentioned earlier on; in other animals, the circular coat showed throughout the experiment powerful outbursts of rhythmic

activity, which occasionally were so persistent as to make the observation of the response to drugs impossible.

Two groups of substances were tested on the circular coat of the rabbit's intestine: drugs which act by stimulating the nervous structures and compounds with a direct muscular action. Lactate, eserine, hypertonic sodium chloride, and nicotine were selected as nervous, muscarine and potassium chloride as muscular stimulants.

Lactate. Feldberg & Solandt [1942] have shown that the rabbit's intestine is not capable, as is the heart, of using lactate as a source of energy; accordingly, its effect on the longitudinal coat depends on the presence of glucose. The circular coat, however, is stimulated by lactate even when the longitudinal muscle has become completely quiescent owing to lack of carbohydrate, and the site of the effect, as shown in a previous paper [Vogt, 1943], appears to be Auerbach's plexus. The amount of sodium lactate required for the production of a rhythmical outburst of activity is so large that it cannot be added to the suspension fluid without causing an increase in tonicity sufficient in itself to provoke constriction of the circular coat; a solution was, therefore, prepared in which the sodium chloride of the Tyrode's solution was replaced by an isomolar amount of sodium lactate; this was brought to bath temperature and the required volume added to the half-emptied bath. Examples of the normal responses are found in earlier publications [Feldberg & Solandt, 1942, Fig. 16; Vogt, 1943, Fig. 5]. The doses used were 300, or rarely, 450–525 mg. lactate in a 75 ml. bath. At least partial exhaustion of the longitudinal coat in the glucose-free Tyrode's solution was awaited before the lactate was given. The response was tested in nineteen animals suffering from severe deficiency. The results are seen in Table 3.

TABLE 3. Response of rabbit's intestine (circular muscle) to sodium lactate in suprarenal deficiency

No. of rabbit	Days since completion of supra-renalectomy	Response to lactate	No. of rabbit	Days since completion of supra-renalectomy	Response to lactate
53	1	+	20	9	−
68	1½	+	47	10	−
30	4	±	76	11	+
59	4	+	64	11	−
84	5	+	50	12	−
82	6	+	31	15	+*
22	6	−	54	19	−
83	6	−	65	37	−
33	7	+*	34	59	−
78	8	+			

* Disappearing later.

It is obvious from the table that, the longer the deficiency takes to develop, the more frequent is the absence of a response to lactate; in two rabbits

(33 and 31) the reaction could be elicited when the gut had been freshly removed from the abdomen, but had disappeared in strips which had been kept for 3 hr. in Tyrode's solution at room temperature. In the normal intestine such treatment does not impair the response to lactate; an example is shown in Fig. 5 of the paper on the normal intestine [Vogt, 1943]: the strip had been kept for 4 hr. at room temperature before being used for the record reproduced in the figure. The disappearance of the response in rabbits 31 and 33 is, therefore, likely to be correlated to the suprarenal deficiency and to be the expression of a slighter damage than that apparent in the nine animals in which the contractions were absent from the beginning. Even that mild damage was, however, not seen in the three rabbits 82, 78 and 76; these, in spite of a fairly long period of deficiency, exhibited a normal reaction not only in the fresh strip but also in a second strip which had been kept in Tyrode's solution for 3 hr. before being transferred to the warm, oxygenated bath.

Eserine. In spite of the fact that eserine acts by allowing the acetylcholine in the gut to accumulate until its concentration is sufficient to stimulate the muscle cells, it is dealt with among the substances which excite the nervous tissue in the intestinal wall. This is justified as there is evidence that the acetylcholine is produced by Auerbach's plexus, which is, therefore, responsible for the action of eserine.

Small doses of eserine sulphate (10 μg. in 75 ml.) cause powerful contractions of the circular coat in the normal gut suspended in glucose-free Tyrode's solution. The latent period varies between 1 and 3 min. The effect is not abolished by keeping the intestine for several hours in Tyrode's solution at room temperature before suspending it in the bath.

The response was normal in thirteen suprarenalectomized (deficient) rabbits; it was weak, with latencies up to 5 min., in three, had disappeared in strips kept for 3 hr. at room temperature in two and was entirely absent in another two animals, in which lactate had also failed to elicit any contractions.

Sodium chloride. Hypertonicity of the bath produces rhythmic activity of the circular coat of the normal intestine by stimulation of Auerbach's plexus [Vogt, 1943]; the simplest way of testing this response is by the addition of sodium chloride to the organ suspended for some time in glucose-free Tyrode's solution. 160 mg. NaCl in a 75 ml. bath was the dose chosen, and seventeen deficient animals were used. The response was present in all but two cases; these were two rabbits in which lactate was also without effect.

Nicotine. Nicotine in a dose of 0·3 mg. (added to a 75 ml. bath) is a powerful stimulant of the circular coat of the normal intestine, exhausted by prolonged activity in a glucose-free medium; it is equally powerful in the deficient rabbit: of fifteen animals in which nicotine was tested, the response was poor only once.

Muscarine. The effect of natural muscarine on the circular layer of the normal intestine has been described by Feldberg & Solandt [1942]; it persists in spite of glucose lack and is produced by direct stimulation of the muscle. The response to 0·4–0·5 ml. of a solution 1 in 500 of the available preparation (added to a 75 ml. bath) was examined in eleven rabbits suffering from suprarenal deficiency; it was always present and was normal in size. An example is shown in Fig. 7.

Potassium chloride. A very similar effect to that produced by muscarine on the circular coat is obtained by potassium chloride. 40–100 mg., injected into a 75 ml. bath, produce rhythmic or tonic contractions illustrated, for the normal rabbit, in Figs. 1 and 2 of a previous paper [Vogt, 1943]. The same doses were given to strips of twelve deficient rabbits; no abnormal responses were encountered. The contractions following a dose of 60 mg. are represented in Fig. 8.

Attempts at restoring the lost responses. Several methods were tried in order to restore the response to lactate in pieces of intestine in which it was absent. Addition of 0·1–0·3% 'Eucortone' (Allen and Hanburys) to the warmed oxygenated bath in which the intestine was suspended did not improve its responses. In order to allow a prolonged action of the cortical extract before the gut had spent its energy reserves in spontaneous activity, pieces of intestine were immersed for several hours at room temperature in Tyrode's solution containing 'Eucortone'; after this treatment the gut was suspended in the warm organ bath and kept under the influence of 'Eucortone' till the spontaneous activity had sufficiently subsided to allow the effect of lactate to be tested. This, again, was unsuccessful. Finally, however, a third procedure was adopted, nearly identical with the second, but differing from it in that the solution containing the gut was kept stirred and oxygenated by a current of air. Control pieces were subjected in a similar way to immersion in aerated 'Eucortone'-free Tyrode's solution. Under such circumstances, a response to lactate which had been absent in freshly excised strips was repeatedly observed; this, however, occurred independently of the presence or absence of 'Eucortone' in the aerated solution.

DISCUSSION

The answer to the question put at the beginning of this paper, whether the intestinal musculature of a rabbit dying from suprarenal deficiency shows an abnormal carbohydrate metabolism, is in the negative. The longitudinal coat exhibits the usual spontaneous activity, and only in some deficient animals was the time required for 'exhaustion' by lack of glucose in the suspension fluid shortened; in other words, the tissue reserves of substances capable of providing the energy for the muscular activity were not as a rule subnormal. Like the normal intestine, the gut of the deficient animals metabolized glucose

and pyruvate and was unable to utilize lactate. Moreover, since glycolysis involves phosphorylation, these results afford no indication, in suprarenal deficiency, of a faulty phosphorylation, as postulated by Verzár [1939]. On the other hand, Girndt's [1925] finding that the choline content of the gut is not diminished by suprarenalectomy, at least within the first 24 hr., is of interest in connexion with the observation of normal pendulum movements. Finally, as in the normal intestine, glucose is utilized as the source of energy for contractions of the longitudinal coat elicited by drugs.

No indication could be found of a consistent disturbance in the conversion of endogenous protein into carbohydrate, since only a single case of hypersensitivity to the inhibiting action of phloridzin and iodoacetate was seen. As these substances prevent the cleavage of carbohydrate, their exaggerated effect might have been interpreted as an inability of the tissue to perform work by means of the breakdown of other compounds. The observation that their action was nearly always the same as is the normal tissue, does not disprove, but renders unlikely, the assumption that conversion of protein into carbohydrate is impaired in the gut of the deficient animal.

Whereas the response of the longitudinal muscle to stimulating and inhibiting drugs was invariably normal, some pathological phenomena were found in the circular layer. The tendency to spontaneous activity, both of a continuous and of a rhythmic character, was abnormally high, whereas the response to certain drugs was diminished or absent in a large number of animals. Of the substances tested, those acting on the muscle itself (muscarine and potassium chloride) elicited normal responses in all animals. Drugs whose site of action is Auerbach's plexus, however, occasionally failed to cause any contraction in doses which invariably affect the normal intestine. The number of animals in which the response was absent or weak is shown in decreasing order in the following list:

Lactate	11 rabbits	Sodium chloride	2 rabbits
Eserine	7 rabbits	Nicotine	1 rabbit

This order is the same as that found [Vogt, 1943] for the disappearance of the responses caused by keeping normal intestine for several days at a temperature just above freezing-point: nicotine is the last and lactate the first to lose its efficacy. Drugs which stimulate the muscle (muscarine and potassium chloride) remain active even longer, just as their action is not interfered with by suprarenal deficiency. We come to the conclusion, therefore, that in suprarenal deficiency Auerbach's plexus gradually becomes less excitable, and that responsiveness to different drugs diminishes in the same order as it does when exposure to cold causes slow death of the tissue. This analogy with death from an unspecific cause raises the question whether these changes are a direct consequence of the lack of suprarenal hormone, or whether they are

merely a sign of impending death due to the impaired circulation to which the sensitive nervous structures succumb more rapidly than the musculature. One way in which an attempt was made to answer this question was to try and restore the intestinal responses by cortical extracts. In some instances, such a restitution was, indeed, obtained, provided the 'Eucortone' was supplied in an oxygenated solution. Occasionally, however, recovery also occurred as the result of treatment with oxygen in the absence of 'Eucortone'. Any conclusions from the few successful experiments can only be accepted with reserve, but it seems not improbable that the decisive factor in the treatment was the oxygen and not the cortical hormone.

Although the similarities between certain responses displayed by a piece of normal intestine preserved in the ice-chest and a strip which has been freshly excised from a suprarenalectomized animal are interesting, it is important to remember that in many respects the damage produced by cold is entirely different from that caused by suprarenal deficiency. In the latter, for instance, the perfectly normal behaviour of the longitudinal coat contrasts strikingly with the complete quiescence of the same muscle, if the cooled preparation is suspended in glucose-free Tyrode's solution. Nor does every pathological condition in the living animal affect the intestine in the manner described for suprarenal deficiency. An intestinal strip taken from a rabbit suffering from peritonitis, for instance, had nearly lost the activity of its longitudinal coat and showed very little improvement with glucose, but responded to $10\mu g$. eserine with powerful contractions of its circular muscle. We have seen before that a suprarenalectomized rabbit, on the other hand, may lose the circular muscle's response to eserine, but never shows impairment of the function of the longitudinal coat.

In the interpretation of the results described in this paper, several facts will, therefore, have to be borne in mind. The first is the appearance, in the intestine of suprarenalectomized and severely deficient rabbits, of certain pathological phenomena which are apparently specific for that condition. The second is the absence of any abnormal response unless the rabbit has survived the removal of the second gland for at least 5 days. The third is the resemblance between the failure of the circular coat to react to drugs stimulating its nervous plexus, and a similar failure produced by keeping excised normal intestine at low temperatures. The fourth is the inefficacy of cortical hormone to restore the functional loss of the isolated tissue. These facts are best explained by the assumption that the pathological phenomena observed in the gut are consequences of the disturbances produced in the whole organism by the absence of suprarenal secretion, and are not primary effects of lack of hormone on the intestinal tissue. Neither the metabolic disturbances seen in the suprarenalectomized animal nor its increased susceptibility to the toxic effect of drugs are reflected in the reactions of the isolated intestine.

Summary

1. Of sixty-one suprarenalectomized rabbits, one-half developed 'chronic' deficiency, ending in death. The survival time ranged from 1 to 59 days. Among the signs exhibited by these animals were visceral atrophy and spastic contraction of the small intestine.

2. Isolated strips of the jejunum of the chronically deficient rabbits were suspended in glucose-free oxygenated Tyrode's solution when there were indications that death was imminent.

3. The spontaneous activity of the longitudinal coat was normal in character; its duration was shortened in some of the animals.

4. The circular coat frequently developed powerful spasmodic rhythmic contractions not encountered in the normal controls.

5. The intestine utilized glucose as well as pyruvate. In the longitudinal muscle, glucose restored both the spontaneous activity and the contractions induced by drugs; lactate was not used as a source of energy and required the presence of glucose for its action. All these reactions are the same in the deficient and the normal animal.

6. Phloridzin and iodoacetate displayed an unusually powerful inhibition only in one instance.

7. The responses of the longitudinal coat to stimulating and to inhibitory drugs were normal. In addition to the substances discussed with regard to their effect on the circular coat (see below), responses to histamine, acetylcholine, adrenaline and ephedrine were examined.

8. Substances causing contraction of the normal circular coat by action on Auerbach's plexus often failed to do so in the deficient animal. The response to lactate was most frequently absent, that to eserine less often, and that to hypertonic solutions or to nicotine was only rarely affected.

9. Drugs stimulating the muscle itself (muscarine, potassium chloride) invariably elicited normal responses of the circular layer.

10. The abnormal functions were not restored by cortical extracts. They are interpreted as secondary effects of suprarenalectomy caused by primary deficiencies in other organs.

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AN EFFECT OF BARBITURATES ON
SERUM CHOLINESTERASE

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Several papers have been published on the cholinesterase activity of serum in various physiological and pathological conditions [Hall & Ettinger, 1937; Hall & Lucas, 1937; Stedman, 1935; Antopol, Tuchman & Shifrin, 1937; Jones & Stadie, 1939] and a number of inhibitors of the enzyme has been studied [Roepke, 1937]. Barbiturates were found to have no effect on the cholinesterase *in vitro* [Bernheim & Bernheim, 1936], but no investigation has been reported of a possible effect of prolonged administration of these drugs. Since many drugs of this group are slow acting and are often given for long periods of time, it seemed justifiable to investigate whether they had any effect *in vivo*.

The present paper describes the decrease of cholinesterase activity of serum after prolonged administration of two barbiturates in human beings; no evidence could be found for it being due to the action of an inhibitor of the enzyme. It is therefore inferred that the decreased activity is due to an actual diminution of the amount of enzyme present.

METHODS

From several young and healthy subjects blood was taken from a vein six or eight times during one day, and from a number of guinea-pigs by heart puncture four times daily. This was repeated after a few days. It was found that the cholinesterase activity of serum from blood taken in the morning (before breakfast, or, in the case of the guinea-pigs, after they were kept fasting over night) was nearly always the highest, and was reasonably constant in the same individual.

For the estimation of cholinesterase, the Hall & Lucas [1937] modification of the continuous titration method of Willstätter, Kuhn, Lind & Memmen [1927] and Knaffl-Lenz [1923] was used. The serum (0·5 ml.) was added to 10 ml. of CO_2-free water in a large test-tube (70 ml.) immersed in a water bath at 37° C. ($\pm 0·1$). A similar test-tube containing phosphate buffer (*p*H 8)

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and the same quantity of the same serum was placed very near in the same water bath. Four drops of 0·4% cresol red (made up without alcohol) were added as indicator to each tube. When the contents of the tubes had reached the temperature of the water bath, 25 mg. acetylcholine chloride in 1 ml. were added to the contents of the first tube. A continuous addition of 0·01 N NaOH to this tube, at just the rate to keep the colour in both tubes equal, was started as soon as possible (i.e. within 30 sec.) after the substrate had been added to the enzyme in the first tube. The burette was read every few minutes. These readings were plotted against time (min.) and the slope of the curve for the first 10 min. starting from the first reading was determined graphically. The curve for the first 10 min. is usually perfectly straight. The slope of this curve appears to be a most accurate expression of the cholinesterase activity of serum and is referred to in this paper as the 'rate of hydrolysis'. The non-enzymic rate of hydrolysis of the same amount of substrate under the same conditions was found to be 0·020, as an average of six determinations. Because this value is small and would not influence any of the conclusions drawn in this paper, it was neglected.

A small glass stirrer of the ring type was used in the vessel where the enzyme reaction took place, but was lifted out frequently for a few seconds when the colours in the vessels were compared. The vessel was closed by a cork into which the lower end of the burette was fixed. The heating lamp in the water bath was screened and the titrations were carried out by daylight. A sheet of paper painted with the complementary colour (greenish yellow) to the reddish blue colour of the indicator (at pH 8·0) was placed nearby, so that the eye could rest on it at intervals. This was thought to increase the accuracy of the colour perception.

Since it was found that some of the bactericidal fluids in which the needles for venous puncture are sometimes kept were potent inhibitors of the cholinesterase, only clean boiled needles were used. Care was taken to avoid haemolysis since this disturbs the colour readings. 1–2 hr. after the blood was taken, the clot was detached and centrifuged. The activity of the sera was usually determined on the same day; sometimes this was postponed to the following day, the serum being kept in the ice chest (+4° C.). This delay did not influence the activity to any appreciable extent.

<div align="center">RESULTS</div>

<div align="center">*Decrease of cholinesterase activity after barbiturates*</div>

When it was found that single and comparatively large doses of phenobarbitone soluble (40 mg. subcutaneously) in guinea-pigs, and 80 mg. orally in two healthy young subjects, and phenyl-methyl barbituric acid (Rutonal) in single doses of 195 mg. in human beings, had no obvious effect on the cholinesterase activity of the serum, 2, 8 and 24 hr. aft— ̣ ̣ ̣ ̣ had been

given, experiments were carried out in order to find out whether prolonged administration in doses not causing obvious narcosis had any influence.

Since the majority of patients suffering from epilepsy are to-day treated with barbiturates, the sera of a number of such patients were assayed. The results are shown in Table 1. In order to enable one to distinguish between the action of the barbiturates and the eventual effect of the disease or the epileptic fits, and since there are very few epileptics under no treatment to-day, some patients suffering from other mental diseases were selected, and the same barbiturate treatment was given to them as to the epileptics for varying periods of time. The values obtained from these non-epileptics, when treated with the same drug for about 21 days, were found not to differ significantly from the average values obtained from epileptics (see the last four values given in column 1 of Table 1). All persons of column 1 were treated for at least 3 weeks, and most of them for much longer periods, with phenyl-methyl barbituric acid (Methophenobarbital); most of them received twice daily a dose of 195 mg., a few of them receiving one such dose in the morning and double this dose in the evening. Some of them had been treated with methyl-ethyl-phenyl barbituric acid (Phemitone) some months earlier. In column 2 of Table 1 the values are given for patients selected at random, and column 3 gives values for 'healthy persons'.

Blood was taken from all subjects, except blood donors, in the morning before breakfast; from blood donors it was taken in the afternoon, at least 3 hr. after the last meal.

It can be seen from Table 1 that the values from all subjects under barbiturate treatment are considerably lower than those of the controls. Further proof that the reduction found was due to the barbiturate treatment has been provided, moreover, by the repeated determination of the serum activity of a number of patients who were under the treatment for a long time and who were taken off and put again under the treatment [Schütz. 1941].

Tod & Jones [1937], though noting the great scattering of their results obtained from epileptics, found rather low values in such cases. Stedman & Russell [1937] also found low values in epileptics. Table 1 shows that the values obtained from untreated epileptics are not lower than those from other subjects. This is confirmed by the findings mentioned above [Schütz, 1941], when the treatment was discontinued in such patients. It seems very probable, therefore, that the lower values found by Tod & Jones and by Stedman & Russell in epileptic subjects were due to some treatment and have no connexion with the epilepsy.

The values of the standard deviation of the average $\left(\sqrt{\dfrac{\Sigma d^2}{n\,(n-1)}} \right)$ which are given in Table 1, show that the results do not show very great spread. If the average values of columns 1 and 3 are x_1 and x_2, and their respective

standard deviations σ_1 and σ_2, then $\dfrac{x_1 - x_2}{\sqrt{(\sigma_1^2 + \sigma_2^2)}} = 8$, indicating that the result is very significant. The average values of columns 2 and 3 (Table 1) are similar and, if the above-mentioned expression is calculated for columns 1 and 2, it becomes even greater still (> 9).

TABLE 1. Showing cholinesterase activity of serum

$$\text{Rate of hydrolysis} = \frac{\text{vol. of } 0.01\,N \text{ NaOH in ml.}}{\text{time in min.}} \times 10.^*$$

25 mg. acetylcholine chloride, 0·5 ml. serum, pH 8·0, 37·0° C.

Under barbiturate treatment (for at least 3 weeks)		Controls			
		Different cases under no treatment		Students, blood donors, etc.	
E.L.R.	0·726	Duodenal ulcer	1·925	L.D.A.	2·274
G.T.	0·957	Anxiety neurosis	2·230	E.S.	1·529
H.G.	0·753	Epilepsy	2·370	F.S.	1·795
V.O.W.	0·682	Melancholia	2·025	G.L.	2·070
E.L.	0·648	Diverticulitis	1·440	G.H.	2·996
J.H.A.	0·930	Melancholia	2·810	Blood donor	2·045
J.B.	0·582	Schizophrenia	2·595	,,	2·340
W.P.	0·448	Gastric ulcer	1·330	,,	1·172
W.E.P.	0·732	Schizophrenia	1·980	,,	2·560
E.M.R.	0·757	Epilepsy	2·550	,,	1·250
C.C.	0·418	Confusional insanity	1·740	,,	2·340
C.P.	0·781	Insanity with fits	1·225		
C.J.	1·052	Epilepsy	2·413		
M.A.P.	0·967				
S.B. (melancholia)	0·546				
W.M.F. (schizophrenia)	0·588				
P.F. (schizophrenia)	0·798				
M.W. (melancholia)	0·490				
Average ($n = 18$)	0·714	($n = 13$)	2·048	($n = 11$)	2·088
S.D.M.	±0·043		±0·140		±0·168

* The actual rates have been multiplied by 10, in columns 2 and 3.

Experiments in vitro

Addition of the drugs to serum. After it was found that the two barbiturates caused, after prolonged treatment, a decrease of the cholinesterase activity of serum, the question arose whether this effect was due to an inhibition of the enzyme. Bernheim & Bernheim [1936] found that phenobarbitone in $0.001\,M$ concentration had no inhibitory effect on the cholinesterase activity of brain *in vitro*. Since this question of direct inhibition is important for the interpretation of the results described here, some *in vitro* experiments were carried out, using two barbiturates and various concentrations of the drugs.

Sera of normal subjects were mixed (1 : 1) with solutions of phenobarbitone from 0·5 to 4 % (0·02 to 0·16 M) and left standing at room temperature. Since the drug gives a strongly alkaline solution, equal quantities of the same serum mixed with 0·9 % NaCl solution (1 : 1) were brought to the same pH with NaOH as the barbiturate serum mixture (pH 8·3). The activity of both solutions was determined using the equivalent of 0·5 ml. of serum. Since the

action of some inhibitors is known to take place slowly, and since it is known that (for example with eserine) the cholinesterase requires 8 hr. to reach equilibrium [Roepke, 1937], the mixtures were left standing for different times up to 6 hr. at room temperature and thereafter for 18 hr. in the ice chest before the determination was made. The other barbiturate used (Methophenobarbital) is scarcely soluble in water, but it was found eventually that up to 2·3 mg. are soluble in serum itself at room temperature. To controls, an equimolar amount of NaCl was added. In none of these cases could any difference be detected between the activity of the sera to which the drug had been added *in vitro* and the controls. In the case of the strongly alkaline phenobarbitone and the alkaline control serum mixture, a slight decrease was evident after 22 hr., but it was equal in both and probably due only to the alkalinity of the mixtures.

From these experiments it was clear that the described effects of the drugs *in vivo* could not be explained by a direct inhibiting action of the drug on the enzyme. These experiments could not, of course, exclude the possibility that the drug being decomposed (detoxicated) in the organism, could at some state be converted into an inhibitor, or cause the formation, or increase the amount, of such an agent. Some further experiments were therefore carried out which could show the presence of an inhibiting substance in those sera which were obtained from subjects to whom a slow-acting barbiturate had been given over a long period. When normal sera (from man or horse) with their usual high cholinesterase activities were mixed with sera from subjects under barbiturate treatment, no reduction in the activity of the former by the latter was observed.

Dialysis of sera. Some enzyme inhibitors are known to be removable from the enzyme by dialysis, like eserine from cholinesterase [Matthes, 1930]. Stedman & Russell [1937] have shown that the inhibitory effect of prostigmine on the activity of corpuscles disappears during the process of washing. Sera from subjects who were given barbiturates were therefore dialysed for periods up to 30 hr. in the ice chest, in collodion or cellophane against distilled water saturated with chloroform. The distilled water was changed from 10 to 14 times during that period. No increase of activity could be observed, even if the concentrated dialysate of normal serum was added to the dialysed serum in order to replace any activators lost by dialysis [Nachmansohn & Lederer, 1939].

Shape of titration curves. The most accurate way of investigating whether an inhibitor of a competitive type is present is the determination of the dissociation constant of the cholinesterase-acetylcholine complex. The value of determining this constant in unpurified sera, however, is doubtful, and, since it is not known what inhibitors and possible activators of the enzyme reaction would be removed during the purification, no attempt was made to

obtain information in this way. It appears, however, that a strong indication for the presence of an inhibitor could be obtained by an analysis of the kinetics of the titration curves themselves, or by a quantitative estimation of the decrease (or increase) of the rate of hydrolysis with time.

It is well known that the rate of the enzyme-substrate reaction decreases more or less quickly with time according to the amount of substrate present at any given time. In many cases it is not only the decrease of the amount of substrate which is responsible for this decrease in the reaction rate, but also the fact that one or more of the split products are inhibitors of the enzyme-substrate action. In the case of the enzymic hydrolysis of acetyl-choline by choline esterase, one split product, namely choline, is known to be an inhibitor [Roepke, 1937]. The decrease of the rate of the enzyme hydrolysis of acetylcholine with time can, therefore, be attributed to the gradual decrease of the amount of acetylcholine present, and to the gradually increasing amount of choline set free during the reaction. If the enzyme which is added to an excess amount of acetylcholine (as is the case in my determinations) were inhibited by an inhibitor of the competitive type, the rate of hydrolysis would not be expected to decrease to the same extent as it would if no such inhibitor were present; it could, indeed, even increase first, and later on decrease. This is often observed in enzyme reactions when the excess of substrate present in the mixture slowly displaces the inhibitor from the enzyme.

Experiments were therefore carried out with several normal sera and with sera from subjects and animals to whom barbiturates had been given for a long period. The titration was made as carefully as possible and for a longer period (20–40 min.) than was done usually. Smaller quantities of substrate were used so that the presence of an inhibitor of the competitive type could be detected more readily in the way just mentioned. If x is the amount of $0.01 N$ NaOH used in the titrations, and t is time in minutes, the rate of hydrolysis (activity of serum) is expressed in this paper with the first differential quotient dx/dt for the period of the first 10 min. The mathematical expression which can show the presence of an inhibitor of the competitive type is the second differential quotient d^2x/dt^2. In the case of the presence of a competitive inhibition, this should become zero, or even positive, or at least should be of a significantly lower order of magnitude than that obtained with normal or control sera. Relatively smaller amounts of the normal sera were added to the substrate in order to obtain approximately the same rate of hydrolysis as with the sera from the subjects who were under the influence of barbiturates. The gradual decrease of substrate, and the consequent gradual increase of choline, was therefore much the same in both cases. Any pronounced difference in d^2x/dt^2 would thus be significant. X was plotted against time and the values of dx/dt were obtained graphically in the usual way at points corresponding to 3, 9 and 15 min. after the substrate was added to the

enzyme. From these figures d^2x/dt^2 was calculated. Typical results of such experiments are shown in Table 2, from which it can be seen that the values of d^2x/dt^2 are of the same order of magnitude in all cases. They certainly never became zero or positive. .

TABLE 2. Showing the values of d^2x/dt^2 found with control sera and with sera from patients under barbiturate treatment, or with the drug added *in vitro* to normal serum

Rates approximately equal; $x=$ml. $0.01 N$ NaOH; $t=$min.

| | | Control 5 mg. acetylcholine chloride with 0.2 ml. serum | | | Barbiturates 5 mg. acetylcholine chloride with | | |
		Blood donor	J.H.A.	E.C.	0.2 ml. normal serum. Drug added *in vitro*	0.5 ml. serum A.M.M.	H.W.G.
$(d^2x/dt^2) \times 10^3$	$t=6$	-1.73	-2.18	-1.21	-4.33	-3.57	-1.30
$(d^2x/dt^2) \times 10^3$	$t=12$	-1.70	-1.10	-0.50	-1.48	-1.00	-1.20

From these results it appears very unlikely that the low activity resulting from the administration of the drug *in vivo* could be due to the presence of a competitive inhibitor.

Although eserine and prostigmine are also competitors of the substrate for the enzyme [Easson & Stedman, 1936] their inhibiting action seems to be due mainly to a non-competitive action on the enzyme, since this action is not altered by the amount of substrate present [Roepke, 1937]. Though the presence of such an inhibitor in my experiments appears to be unlikely, there remains, of course, the possibility that, although I could not find any inhibiting action of the drug on the enzyme *in vitro* using unpurified serum, an inhibiting action of the drug would appear when a purified enzyme preparation was used. Such an inhibiting action however could not be so powerful, and it is unlikely that the great decrease I have observed could be attributed to such an agent.

Considering these facts it seems that the low cholinesterase activity found in the serum after prolonged administration of barbiturates can be attributed to true diminution of the amount of the enzyme present rather than to an inhibition of the latter.

Quantitative estimation of the decrease of cholinesterase;
equivalent amount of enzyme present

The values given in Table 1, though showing a significant decrease of the cholinesterase activity, do not, of course, enable a correct estimation of the extent of this decrease to be made. Though there exist mathematical relationships between the amount of cholinesterase present and the rate of hydrolysis of acetylcholine, which resembles the type of a unimolecular reaction [Matthes, 1930], it was thought desirable to investigate this question separately for the particular material used (unpurified human serum).

Different quantities of several sera were added to the usual amount of acetylcholine chloride and the activity determined in the usual way. If the values of the rate of hydrolysis found with these different quantities were plotted against the amount of serum, a characteristic curve was obtained. The nearest approach to a straight line was obtained when the logarithm of the rate (r) was plotted against the logarithm of the amount of serum (c). In this case a nearly perfect straight line was obtained which could be expressed by the equation $r = c^{0.739} \times 0.396$, the constants having been determined graphically. From Table 3 it can be seen that the values of the calculated rates of hydrolysis do not differ by more than 7 % from the observed values.

TABLE 3. Showing rates of hydrolysis of acetylcholine chloride (25 mg.) by different quantities of serum from one normal subject (pH 8.0 and 37° C.)

ml. serum	Rate of hydrolysis (found) ml. 0.01 N NaOH/min.	Rate calculated
1.0	0.422	0.3963
0.5	0.255	0.2375
0.25	0.1425	0.1423
0.125	0.0802	0.0852

Since the rates given in Table 3 cover the whole range found with 0.5 ml. of all the human sera hitherto examined, the above equation could serve well for the estimation of the equivalent amount of enzyme present in any particular serum.

Applying it to the results given in Table 1 and designating the average equivalent amount of the enzyme present in the sera of healthy persons (Table 1, column 3, average rate found 0.2088) to be 100 %, then only 22.6 % was found as an average in sera of persons to whom the stated barbiturate was given for a prolonged period of time (Table 1, column 1, rate found 0.0714). The decrease, therefore, must be regarded as considerable.

Negative results

In the course of the investigation described above a number of experiments was carried out in order to discover other possible sources of error or to find other factors which might influence the cholinesterase activity of serum. None of the conditions mentioned below was found to alter the cholinesterase activity of serum to an appreciable extent. Anaphylactic shock (two rabbits and two guinea-pigs), Leptazol convulsions (two rabbits), hyperventilation (two men), 4 min. standing running (two men), lack of sleep (one subject kept awake for 42 hr.), and single doses of NaBr (4 g.), amphetamine sulphate (10 mg.) and caffeine (0.4–0.6 g.) in men.

Discussion

The decrease of cholinesterase activity resulting from the administration of barbiturates appears to be due to a true diminution of the amount of enzyme present. The simplest explanation for this phenomenon seems to be that the cholinergic system under the influence of the narcotic produces a relatively lower amount of acetylcholine, that consequently the need for the enzyme decreases and the latter diminishes in quantity. Under this assumption the cholinesterase might well be looked upon as providing an indication of the average level of activity of the cholinergic system of the particular individual.

This explanation finds ample support in the values found by other authors. Higher enzyme activity was found in states of anxiety [Jones & Stadie, 1939; Tod & Jones, 1937], in beri-beri [Antopol, Glaubach & Glick, 1939]. Lower activity is now definitely ascertained to exist in cases of myasthenia gravis [Stedman & Russell, 1937] in cases of stupor, advanced phthisis and carcinoma [Jones & Stadie, 1939; Tod & Jones, 1937]. In all these cases a permanent change in the average level of muscular activity has taken place. Tod & Jones [1937] arrived at a conception not dissimilar to the one expressed above, and pointed out that the two opposite mental states of anxiety and stupor have accordingly a higher and a lower cholinesterase activity of serum than normals. They report also low values in cases suffering from epilepsy. This would not agree with their explanation but, as already mentioned, the values found by these authors in epilepsy, as well as the low values reported in the same disease by Stedman & Russell [1937], might perhaps have been due to some treatment.

It is apparent that the cholinergic system is normally remarkably stable, and that cholinesterase activity remains unaltered by only transient changes in bodily activity. Repeated injections of acetylcholine, for instance, have no influence on the cholinesterase activity of dogs' serum [Hall & Ettinger, 1937], nor is it altered by injections of adrenaline in doses sufficient to alter the blood pressure [Antopol *et al.* 1939]. Since the very transient effect of both these drugs is well known, it is not surprising that single injections at relatively long intervals are not able to alter permanently what might be called the average level of activity of the cholinergic system, and that injections of short acting substances are therefore not followed by a change in the enzyme system. Emmelin [1939] discovered that some indifferent narcotics like the slow-acting barbiturates, Phenobarbitone soluble, etc., markedly increase the action of acetylcholine on the rectus muscle of the frog, and that this increased sensitivity is not due to an inhibition of the cholinesterase by physostigmine. Whether or how this interesting fact is involved in the decrease of the enzyme after prolonged barbiturate treatment, which is described in this paper, is not yet possible to state.

SUMMARY

1. The serum cholinesterase activity of blood when taken in the morning from fasting individuals was found to give the most characteristic and repeatable values for any particular individual.

2. It was found to be much reduced after prolonged administration of Phenobarbitone soluble or phenyl-methyl barbituric acid, while it appeared unaltered after a single large dose of these drugs.

3. Some *in vitro* experiments are described, from which, as well as from the shape of the titration curves, it appears most likely that this decrease is due to a true diminution of the amount of enzyme present rather than to the action of an inhibitor of the competitive or non-competitive type.

4. The average decrease found in sera of human beings was estimated by means of a characteristic curve constructed for unpurified human serum, and found to be equivalent to a reduction of the amount of enzyme present to approximately 22% of the average amount of enzyme present in untreated individuals.

5. Various acute conditions (anaphylactic shock, Leptazol convulsions, severe muscular exercise, over-ventilation, lack of sleep) were all found not to alter the cholinesterase activity of serum to an obvious extent, nor had single large doses of NaBr, caffeine, or amphetamine any obvious effect. The simplest theory to account for these results is discussed.

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THE EFFECT OF BARBITURATES ON THE CHOLIN-ESTERASE IN DIFFERENT TISSUES

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In the foregoing paper [Schütz, 1943] an effect is described of the prolonged administration of barbiturates on the cholinesterase activity of serum. This was found to be reduced after the drug had been given for some time, and further experiments showed that the reduced activity was due to a decrease of the amount of enzyme present in the serum, rather than to the action of an inhibitor.

Stedman & Russell [1937] have ascertained that in cases of myasthenia gravis the values of the cholinesterase activity of serum are lower than normal. They have, furthermore, examined the activity of whole blood and of corpuscles, and have found the values of the latter to be normal. They suggest that the activity of the corpuscles might represent the amount of enzyme present in the tissues, and the lowered serum values in myasthenia gravis might be the first result of an attempt of the body to compensate for its deficiency in acetylcholine by reducing the amount of cholinesterase available for its destruction.

The discovery of the lowering of the enzyme in serum after barbiturates made it possible to investigate directly the cholinesterase in the tissues as well as in the serum, and to ascertain if, in the case of barbiturate treatment at any rate, the lower values in the serum precede the lowering of the enzyme activity in the tissues.

METHOD

The most suitable animals for these experiments were guinea-pigs, because of the high cholinesterase activity of the serum and other tissues. One drug, soluble phenobarbitone B.P., was administered by subcutaneous injections. The controls were injected with an equal volume of saline. A number of animals was kept under barbiturate by oral administration of a water-insoluble barbiturate (phenyl-methyl barbituric acid). This route of administration provides, of course, a less reliable measure of dosage, but it was thought to have the advantage of being less objectionable than very numerous injections.

The dosage, moreover, had to be altered frequently during the course of the treatment, because a uniform and visible effect of the narcotic on the mobility and behaviour of the animals was aimed at. If visible effects were not evident, the dose was slightly increased, or, if an animal was under full narcosis, the dose was slightly decreased. These small alterations could be conveniently carried out by means of the oral administration.

After different periods of time, the animals were killed, and the blood was collected in Petri dishes and the serum obtained in the usual way. Immediately after killing, the brain, spinal cord and a piece of muscle were prepared. The brain (including the medulla oblongata) and the spinal cord were washed under running tap water, and freed from all the visible blood vessels. The 'muscle' was always the entire femoral musculature. The water was allowed to drain off from these three organs and they were cut into fine pieces in separate mortars. Thereafter they were ground thoroughly with cleaned dry sand for at least 10 min. To this paste a few ml. of $M/5$ phosphate buffer (pH 8) were added and well mixed. Gradually more buffer was added until approximately one-third of the total volume appeared still liquid after 15 min. The mixture was then left standing for 1 hr. at room temperature, centrifuged and the supernatant liquid dialysed at $+4°$ C. in collodion tubes under its own pressure against distilled water saturated with chloroform. The dialysis was essential, because phosphates are known inhibitors of cholinesterase, and because the activity was determined by a continuous titration method. The chloroform was added to inhibit bacterial growth. The distilled water was changed at least eight times in 24 hr. Thereafter the activity of a sample of the dialysed extract was determined by means of the continuous titration method of Hall & Lucas [1937] as described in the foregoing paper.

The extracts of the brain and spinal cord were dialysed for 18–24 hr. before the determination, the extracts of muscle for 36–42 hr. One ml. of the dialysed extract was evaporated to dryness on a boiling water bath and the solid residue weighed. The activity was expressed by the number representing the slope of the titration curve (rate of hydrolysis $=$ ml. $0.01 N$ NaOH/min.) interpolated for the first 10 min. after the addition of the 25 mg. acetylcholine chloride in 1 ml. to the extract ($=$rate of hydrolysis, $37°$ C., pH 8·0). In the control vessel, containing the buffer solution (pH 8·0), an equal quantity of extract was added, and in both tubes the same indicator (4 drops of 0.3% non-alcoholic solution of cresol red) was used. The activity of the extract was finally expressed by the 'weight number' which was obtained from the above-mentioned data by means of the following expression: $n = r \times 10/gv$, where n is the 'weight number', $r =$ rate of hydrolysis, $g =$ dry weight per ml. of extract, and $v =$ the volume in ml. used in the determination.

The usual amount of liquid extract obtained after dialysis was 4–5 ml. from one total spinal cord and approximately 8–10 ml. fr

muscle, taken as described above. Usually 3 ml. were used for one determination to act upon 25 mg. acetylcholine chloride.

The activity of the serum was determined by means of the method mentioned in the foregoing paper.

RESULTS

Guinea-pigs were treated with phenobarbitone soluble by subcutaneous injections for 14–18 days. The doses were given thrice daily at intervals of 8 hr. A few times during the whole period of administration the doses were given twice daily at intervals of 12 hr. Approximately 10–16 mg. per animal were given thrice daily, but as mentioned above, more or less was given according to its effect. The effect obtained was very clearly visible; not only were the animals less active than the controls, but they usually had their eyes half-closed and reacted much less to stimuli such as the opening of the cage, etc. If this effect was not obtained, the dose was slightly increased, and conversely, if the animal lay down and reacted only slightly or not at all to stimuli like touch, the dose was decreased or one dose entirely omitted. Three guinea-pigs were treated by oral administration of phenyl-methyl barbituric acid as described above. With these animals a permanent effect on mobility and behaviour was well visible with doses averaging 15 mg. per day. Since it had been previously found that only large, fully grown, guinea-pigs showed significant results after administration of barbiturates, only such animals were used in the experiments described below.

TABLE 1. Guinea-pigs given barbiturates over a period of at least 2 weeks

	Cholinesterase activity expressed in 'weight numbers'			Cholinesterase activity expressed in 'rate of hydrolysis'; 25 mg. acetyl-choline chloride + 0·5 ml. serum
No.	Brain	Spinal cord	Muscle	Serum
1	30·1	40·4	3·42	—
2	38·6	33·8	10·25	0·1098
3	46·5	68·0	11·28	—
4	37·5	45·8	8·17	0·1120
5	32·4	60·0	9·56	0·1976
6	28·0	13·97	6·7	0·1105
7	29·3	52·6	8·5	0·1377
Average	34·6	44·9	8·26	0·1320
S.D.M.	(±2·5)	(±6·8)	(±1·0)	(±0·0175)

The results expressed in weight numbers found in different tissues of these animals are shown in Table 1. The number given for the activity of normal serum is the average of eighteen determinations and represents the 'rate of hydrolysis' of 25 mg. acetylcholine chloride obtained with 0·5 ml. serum at pH 8 and 37° C. Since the sera were thought to be a more uniform material, no dry-weight determinations of these were made, and thus no 'weight numbers' of the sera are given. The results obtained with control animals are given in Table 2.

TABLE 2. Normal guinea-pigs

	Cholinesterase activity expressed in 'weight numbers'			Cholinesterase activity expressed in 'rate of hydrolysis'; 25 mg. acetylcholine chloride + 0·5 ml. serum
	Brain	Spinal cord	Muscle	Serum
	46·1	99·0	11·7	
	38·6	95·0	14·22	
	47·6	102·8	14·3	$n = 18$
	38·7	86·8	14·7	
	41·8	72·8	19·65	
	31·23	96·9	14·6	
Average	40·1	92·2	14·7	0·2104
S.D.M.	(±2·4)	(±4·45)	(±1·06)	(±0·0165)

From Tables 1 and 2 it can be seen that a decrease of cholinesterase activity is evident in the serum, spinal cord and muscle of the animals under barbiturate treatment. A small and probably insignificant decrease was found in the brains. No significant difference between the effect of the two barbiturates used could be ascertained with the small number of animals used.

From Table 3 it can be seen that the average reduction of the cholinesterase activity of spinal cord and muscle extracts is around 50%; that of serum is around 40%. The reduction of the serum activity is thus much less pronounced than that reported for sera of man after barbiturate treatment (see foregoing paper).

TABLE 3. Guinea-pigs: average values of cholinesterase activity

	Cholinesterase activity expressed in 'weight numbers'			Cholinesterase activity expressed in 'rate of hydrolysis'; 25 mg. acetylcholine chloride + 0·5 ml. serum
	Brain	Spinal cord	Muscle	Serum
Normal ($n = 6$)	40·1 (±2·4)	92·2 (±4·4)	14·7 (±1·1)	0·2104 (±0·0165) ($n = 18$)
Barbiturate ($n = 7$)	34·6 (±2·5)	44·9 (±6·8)	8·4 (±1·0)	0·1320 (±0·0175) ($n = 7$)
Decrease after barbiturate treatment (%)	14	51	44	38
Significance of difference	1·5 (<3)	5·8 (>3)	4·5 (>3)	3·3 (>3)

The numbers in Table 3 under 'Significance of difference' represent the value of the expression $\dfrac{x_1 - x_2}{\sqrt{(\sigma_1^2 + \sigma_2^2)}}$, where x_1 and x_2 are the average results of the two groups of animals (treated and controls), and σ_1 and σ_2 the corresponding standard deviations of these average results. The above expression should be at least 3·055 according to Fisher's tables [1925] for the degrees of freedom of the number of our experiments, if the differences are to be regarded as significant. It can be seen that for spinal cord, muscle and serum the differences observed may be regarded as significant, while the values observed with brain extracts from treated animals do not represent a significant decrease.

Further, three guinea-pigs were treated with phenyl-methyl barbituric acid (orally) in the same way as described above and killed earlier after the start of the treatment than the ones previously mentioned. These experiments were carried out in order to obtain some information as to whether the decrease of the enzyme in the serum precedes the decrease in other tissues, and if a 'deranged distribution' of the enzyme could be ascertained in this case. The three guinea-pigs of this series were killed 5, 8 and 11 days respectively after the start of the treatment. The results are given in Table 4.

Table 4. Guinea-pigs treated for different periods. Cholinesterase activity expressed per dry weight of extracts (weight numbers) or as rate of hydrolysis (serum)

Days treated	Brain	Spinal cord	Muscle	Serum
5	38·8	59·1	9·3	0·1866
8	35·0	52·2	10·6	0·1240
11	38·1	60·0	8·5	0·1678
Average from guinea-pigs treated for at least 2 weeks (n=7)	34·6 (±2·5)	44·9 (±6·8)	8·26 (±1·0)	0·1320 (±0·0175)
Average from controls (n=5)	40·1 (±2·4)	92·2 (±4·45)	14·7 (±1·06)	0·2090 (±0·0178)

As can be seen from Table 4, no evidence could be found that the effect upon the cholinesterase developed more quickly in any one of the mentioned tissues than in the others.

Two guinea-pigs not previously treated were brought into deep narcosis by soluble phenobarbitone (60 mg. subcutaneously) and killed 3 hr. after the injection. No reduction was found in any of the four tissues. It can be assumed therefore that only prolonged treatment can evoke the effect. This confirms the findings reported earlier [Schütz, 1941] and in the foregoing paper that single large doses had no effect upon the serum cholinesterase in man.

SUMMARY

1. The prolonged administration of barbiturates causes a fall in the serum cholinesterase of guinea-pigs, though this is much less pronounced than in man.

2. The fall is not confined to the serum, but is evident in the spinal cord and muscle also.

3. No significant fall was observed in the brain.

4. No evidence could be found for a 'deranged distribution' of the cholinesterase, since the reduction started approximately at the same time in each of the examined tissues.

5. The cholinesterase of brain, spinal cord, muscle or serum was not affected by a single deep narcosis induced by one barbiturate.

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THE ANTAGONISM BETWEEN THE POSTERIOR PITUITARY LOBE AND INSULIN

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The antagonism between insulin and the posterior pituitary lobe was clearly demonstrated by Burn [1923] who found that in rabbits the fall of the blood sugar after insulin was counteracted by large doses of posterior pituitary extract. When the effects on blood pressure and uterus were destroyed by treatment with NaOH the anti-insulin action also disappeared.

The attempt to explain the mechanism of this antagonism and to decide the question whether it was due to vasopressin or oxytocin met with many difficulties and led to contradictory results. It seemed, therefore, desirable to investigate again the influence of various extracts of the posterior pituitary lobe on the blood sugar of animals both with and without the administration of insulin.

The effect of posterior pituitary extract on the level of the blood sugar, discovered by Borchardt [1908], can be observed after intravenous or subcutaneous injection of moderate doses. The resulting hyperglycaemia passes off within about an hour. Both vasopressin and oxytocin have been held responsible for this action. Haferkorn & Lendle [1933] and Ellsworth [1935] found in rabbits that the blood sugar rose by 18–40% after vasopressin, and an increase of up to 100% was observed by Thaddea & Waley [1933]. In dogs, Geiling & Robins [1938] found that both extracts raise the blood sugar. In Holman & Ellsworth's [1935] experiments 0·0125 unit oxytocin/kg. corresponded in hyperglycaemic action to 0·25 unit vasopressin/kg. Ellsworth [1936] also found oxytocin the more effective substance, and Houssay & Magenta [1929] came to the same conclusion in their experiments on hypophysectomized dogs. Himwich, Haynes & Fazikas [1932] noted that in normal dogs plasma glucose was increased after injections of pituitrin, pitressin and pitocin, but that it was lowered when the animals were anaesthetized. In unanaesthetized dogs whose femoral vessels were exposed for convenience of injecting and sampling Geiling, de Lawder & Rosenfeld [1931] found that

both pitressin and pitocin increased the blood sugar whether insulin had been given or not.

Other types of antagonism between insulin and posterior pituitary lobe were studied by Quigley & Barnes [1930] and Koref & Mauntner [1926]. The former authors found that the hypermotility of the gut after insulin was counteracted by pituitrin, vasopressin and oxytocin, an effect which partly preceded the hyperglycaemia. The latter investigators published experiments on the inhibition by insulin of the antidiuretic effect of pituitrin.

The antagonism to insulin was considered by Lambie & Redhead [1929] to be possibly due to the influence of pituitrin upon the circulation which could cause a delay in the absorption of insulin from the tissues. The same opinion was held by Wermer & Monguis [1933] who observed that the fall of blood sugar was not inhibited by pituitary extract when insulin was given intravenously. On the other hand, Russell & Cori [1937] concluded from their experiments that hypophysectomy delayed the absorption from the sub-cutaneous tissues. Griffiths [1941], comparing the effect of pituitrin on the blood sugar after intravenous and subcutaneous injection of insulin, came to the conclusion that, as regards intravenous insulin, animals reacted irregularly.

Thaddea & Waley's [1933] findings suggested that the blood-sugar raising effect of pituitrin was due to mobilization of glycogen from the liver. No hyperglycaemia occurred when the glycogen content had been lowered before by poisoning. Corresponding observations in man were made by Thaddea [1933]. Geiling, Campbell & Ishikawa [1927] and Lawrence & Hewlet [1925] believed the antagonism to insulin to be due to an action upon the sympathetic nervous system or the suprarenals, and, consequently, upon the liver.

From their investigations in man, Cohen & Libman [1937] concluded that the antagonism between posterior pituitary and insulin was due to a peripheral action. They observed that pituitrin inhibited the development of the gap between arterial and venous sugar concentration (A.-V. difference) which otherwise appeared after the injection of insulin.

As the different conclusions reached by the various authors could be partly ascribed to the very great variations in dosage and other experimental factors it was decided to examine under standardized conditions the action of pituitrin, vasopressin and oxytocin on the blood sugar of the rabbit after intravenous and subcutaneous injection of insulin.

METHODS

Rabbits which had not been fed for at least 16 hr. were given injections into an ear vein or under the skin. Insulin (Burroughs Wellcome and Co.) and pituitrin, pitressin and pitocin (Parke, Davis and Co.) were used. Insulin and pituitary extracts were given at the same time. Blood sugar was determined by the method of Hagedorn-Jensen.

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L. WISLICKI

Results

While doses of 20 or 40 units of pituitrin when given by themselves caused the blood sugar to rise temporarily (Fig. 1), their effect upon the hypo-
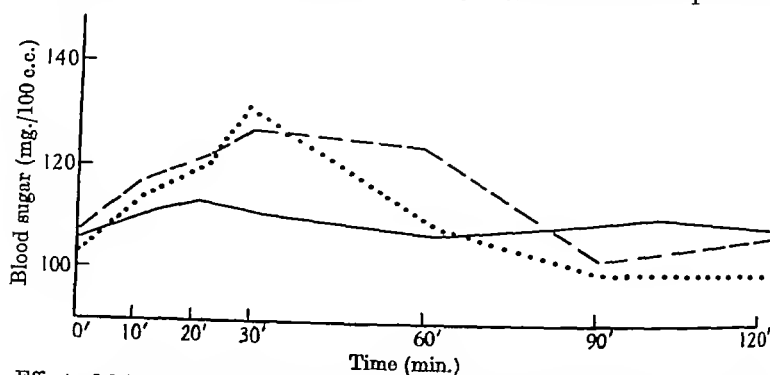
Fig. 1. Effect of different doses of pituitrin on the blood sugar. Rabbit, 2·1 kg. ——— 20 units subcutaneously. ······ 40 units subcutaneously. ——————— 60 units subcutaneously.
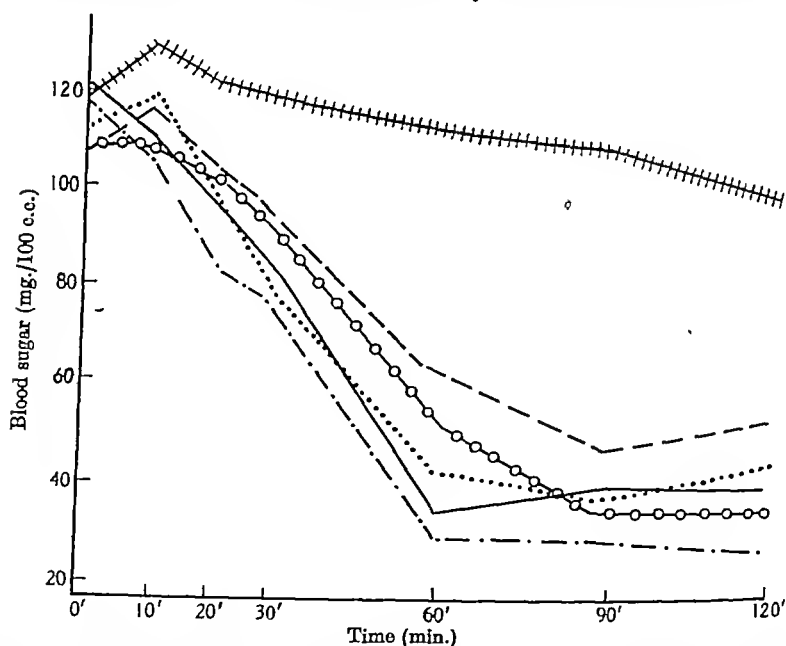
Fig. 2. Effect of different subcutaneous doses of pituitrin on the blood sugar after subcutaneous injection of insulin in small and large doses. Rabbit, 2·3 kg. ——— 3 units insulin. ······ 3 units insulin and 20 units pituitrin. ——————— 3 units insulin and 40 units pituitrin. ⧻ 3 units insulin and 60 units pituitrin. —··—··— 20 units insulin. o—o—o 20 units insulin and 60 units pituitrin.

glycaemia after insulin was uncertain. Usually, 60 units of pituitrin were required to counteract the effect of subcutaneous injection of 3 units of

insulin (Fig. 2). On the whole, consistent results were obtained in ten experiments. In two rabbits the antagonism was incomplete. Their blood sugar fell to some extent, as shown in Table 1.

TABLE 1. Incomplete inhibition of insulin action by pituitrin

Rabbit 1·8 kg.	3 units insulin subcut.	60 units pituitrin subcut.	3 units insulin + 60 units pituitrin both subcut.
Blood sugar (mg./100 c.c.):			
Before	123	117	120
After 10 min.	110	120	118
After 20 min.	89	143	111
After 30 min.	67	138	94
After 60 min.	55	115	98
After 90 min.	58	115	85
After 120 min.	61	109	85
After 150 min.	61	112	87

The power of pituitrin to antagonize the action of insulin was limited to those cases in which only a few units were injected subcutaneously. As Fig. 2 shows the hypoglycaemia was not prevented when 20 units of insulin were given.
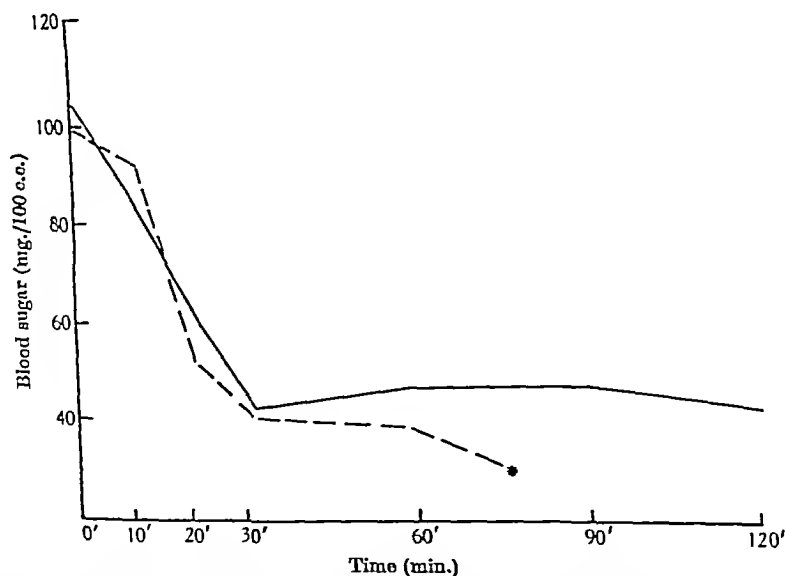
Fig. 3. Effect of subcutaneous pituitrin on the blood sugar after intravenous injection of 3 units of insulin. Rabbit, 2 kg. ——— 3 units insulin. – – – – 3 units insulin and 100 units pituitrin. * Convulsions.

The fall of blood sugar after *intravenous* injection of insulin was not inhibited by pituitrin (ten animals). Even 100 units of pituitrin did not prevent the blood sugar from falling to 40 mg./100 c.c. within half an hour after 3 units of insulin had been given intravenously (Fig. 3).

The large doses of pituitary extract had a marked general effect on the animals. All of them survived, but during the first hour of the experiment they were hyperpnoeic, sprawling their limbs and showing general signs of shock. Such symptoms were not present after subcutaneous injection of 0·25% acetic acid in which pituitrin is usually dissolved (six animals).

TABLE 2. Effect of pitressin and pitocin on the blood sugar

Rabbit 2 kg.	60 units pitressin subcut.	60 units pitocin subcut.	60 units pitressin + 60 units pitocin both subcut.
Blood sugar (mg./100 c.o.):			
Before	108	116	105
After 10 min.	114	116	107
After 20 min.	130	120	118
After 30 min.	133	113	125
After 60 min.	117	118	112
After 90 min.	104	118	110
After 120 min.	107	111	101
After 150 min.	112	113	108
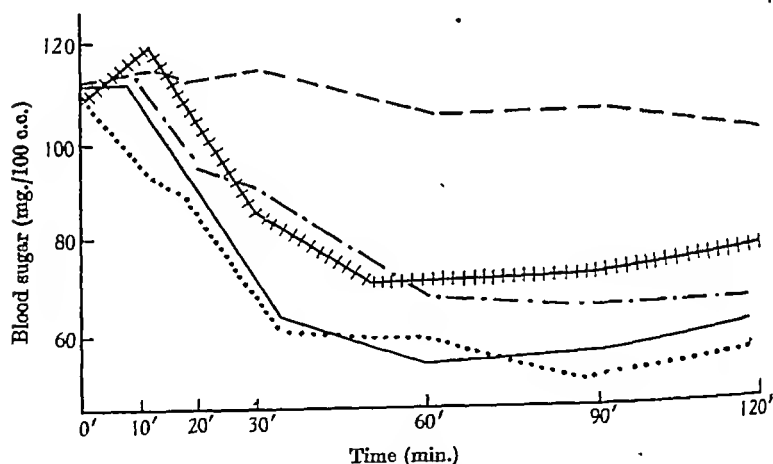
Fig. 4. Effect of subcutaneous pituitrin, pitressin and pitocin on the blood sugar after hypodermic injection of 3 units of insulin. Rabbit, 2·2 kg. ——— 3 units insulin. – – – – 3 units insulin and 60 units pituitrin. ⧺ 3 units insulin and 60 units pitressin. –·–·– 3 units insulin and 60 units pitressin and 60 units pitocin. ······ 3 units insulin and 60 units pitocin.

As the extract of the whole posterior lobe did not antagonize the action of insulin which was injected intravenously, pitressin and pitocin were tested only against insulin when given subcutaneously. When injected alone pitressin caused the blood sugar to rise almost immediately as may be seen in Table 2, while pitocin had no effect. The combination of pitressin and pitocin was not more effective than the vasopressor extract by itself. A subcutaneous injection of 3 units of insulin was partly counteracted by pitressin (Fig. 4), but

when the result was compared with the action of pituitrin it was found (in eight experiments) that the extract of the whole lobe was much more powerful an antagonist to insulin than even pitressin and pitocin together.

DISCUSSION

While our experiments confirm the blood-sugar raising effect of posterior pituitary extract they also show that this action is not responsible for the antagonism to insulin. Large doses cause a passing rise of the normal blood sugar but do not influence its fall after insulin. Even larger doses are required to counteract this hypoglycaemia, and these act only when the dose of insulin is small: 60 units of pituitrin will inhibit the fall of the blood sugar after 3 units of insulin but not after a large dose. Comparing the results obtained after pituitrin and insulin when injected by themselves with those produced by the combined injection, one sees that the inhibition of the insulin hypoglycaemia is not due to the rise of the blood sugar after pituitrin being superimposed upon the fall after insulin.

The antagonism can only be observed when the insulin is given subcutaneously. The failure of pituitrin, even in doses of 100 units, to counteract insulin when injected intravenously, suggests at first sight that the antagonism is due to the effect of pituitrin upon the circulation and, consequently, due to a delayed absorption of insulin from the subcutaneous tissue. Our results show, however, that pitressin has only a slight effect upon the insulin hypoglycaemia. It inhibits the fall of the blood sugar to some extent, but pituitrin in equivalent dosage is a far stronger antagonist to insulin. If the anti-insulin effect of pituitrin were due to its action upon the circulation it might be expected that pitressin would be equally active in counteracting the hypoglycaemia. In actual fact, neither pitressin by itself nor together with a corresponding amount of pitocin is as powerful in this respect as pituitrin.

A difference in the action of insulin after intravenous and subcutaneous injection may be responsible for the finding that pituitrin inhibits the hypoglycaemia after insulin when given subcutaneously but not when injected direct into the blood stream. Macleod [1926] has shown that the fall of the blood sugar after insulin in the first half-hour is steeper when the injection is given intravenously. By the subcutaneous route the effect is slower and less insulin is excreted in the urine. It appears that after an intravenous injection the more acute action of insulin overcomes the antagonism of pituitrin even when the amount of insulin is small.

The slower effect of a small subcutaneous dose of insulin is counteracted by a large amount of posterior pituitary extract. But when a large dose of insulin is given the inhibition is not powerful enough to overcome the hypoglycaemic effect.

SUMMARY

1. Both pituitrin and pitressin in large doses cause a transitory hyperglycaemia in the rabbit. Pitocin is ineffective.

2. Very large doses of pituitrin inhibit the hypoglycaemia which is caused by small doses of insulin given subcutaneously. Pitressin has a slight antagonistic action only, and pitocin has no such action. The fall of blood sugar after large subcutaneous doses of insulin is not inhibited by pituitrin.

3. Insulin when injected intravenously is not antagonized by pituitrin.

4. The inhibition of the hypoglycaemia after the subcutaneous injection of insulin is much stronger after pituitrin than after pitressin and pitocin when injected together in corresponding doses.

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THE INFLUENCE OF THE SYMPATHETIC NERVOUS SYSTEM ON CAPILLARY PERMEABILITY IN TRAUMATIC SHOCK

By D. ENGEL, *From the Department of Physiology, University of Sheffield*

The following two facts have been established in previous papers: (1) If a limb is deprived of its sympathetic nerve supply the permeability of the capillaries is reduced [Engel, 1941]. (2) The rate of filtration through the capillaries in tissue adjacent to that subjected to trauma, and probably also in the traumatized area itself, is increased during the first 1–5 hr. after crushing, and decreased afterwards—a fact which, under certain conditions, indicates increased, and later decreased, capillary permeability [Engel & Forrai, 1943].

From these two observations the question arises whether it is possible to reduce the increased capillary permeability in a crushed limb by depriving it of its sympathetic nerve supply? This question is, apart from theoretical, of eminent practical interest. It is clear that, if the increased permeability in the crushed area is one of the most important initiating factors in producing shock, as is generally assumed, then it should be possible to reduce the liability to and danger of shock by blocking the sympathetic nerve supply of a crushed area. This possibility appears the more within range of practical application as the period of increased rate of filtration lasts only 1–5 hr. after trauma, and blocking the sympathetic chain for this period is a safe and easy procedure. The capillary permeability of crushed limbs, as manifested by the rate of filtration through the capillary wall, after sympathectomy, was the object of this study.

EXPERIMENTAL

Eleven fully grown cats were used in this series. The whole lumbar sympathetic chain and the first sacral ganglion of the right side were removed under ether anaesthesia [see operative technique in Engel, 1941]. The chain thus removed from about 3 cm. above the crus diaphragmatis to below the first sacral ganglion measured 10–14 cm. The animals recovered from this operation in a few hours. 3–9 days later they were used for the experiment: nembutal (for veterinary purposes) was given intramuscularly (0·5 c.c./kg.).

The right, sympathectomized, leg was then traumatized: the soft parts of the calf, avoiding the knee joint, were hammered for 5 min. (100–150 blows with a hammer of 300 g.), and in most experiments also the tibia was fractured at its distal end. Breaking of the skin was avoided. In a series of three animals which were also similarly sympathectomized, both legs were equally crushed and fractured. Both these groups were afterwards similarly treated.

Immediately after trauma 1% acid fuchsin solution was injected into the jugular vein at a constant rate (10 c.c./25 min.) throughout the experiment lasting 3–6 hr., while the two knee joints were simultaneously perfused with Ringer's solution. The knee joint of the non-traumatized leg served as control. The technique of the perfusion and infusion, with their principle, were described elsewhere [Engel, 1941, 1940]. The perfusates of the two knee joints were collected separately every 20–30 min. and their dye content estimated, after addition of a few drops of sulphuric acid, by the colorimetric method.

The animals used in work previously reported served as controls for the present experimental animals. Thirteen cats were traumatized, without previous sympathectomy [Engel & Forrai, 1943], and in fifty-five animals (dogs, cats and rabbits) a study was made of the effect of sympathectomy without trauma on the capillary permeability [Engel, 1941]. In all these experiments the dye concentration was expressed and charted in mm., as read on the colorimeter, the same amount of the perfusate being in each case compared with a standard solution of 1/400,000 of acid fuchsin.

RESULTS

To appreciate the effect of a combination of trauma and sympathectomy on capillary permeability one must remember the effect of each experimental procedure in the absence of the other. Such a reminder is given in Figs. 1 and 2 showing, on the one hand, the increased permeability resulting from trauma, followed after 2–5 hr. by a significant reduction below normal, and, on the other hand, a prolonged decreased permeability resulting from sympathectomy. The permeability of the traumatized side was 2–7 times higher than that of the control side.

Crushing one leg after sympathectomy

Out of eleven animals of this series, one cat was used for perfusion 2 days, four cats 3 days, four cats 5 days, one cat 6 days and one cat 9 days after sympathectomy. In four animals the dye concentration in the perfusate of the traumatized side was less than or equal to the concentration in that of the normal control side, and in six animals it was equal to or inconsiderably increased in some period of the experiment; the only exception will be mentioned later.
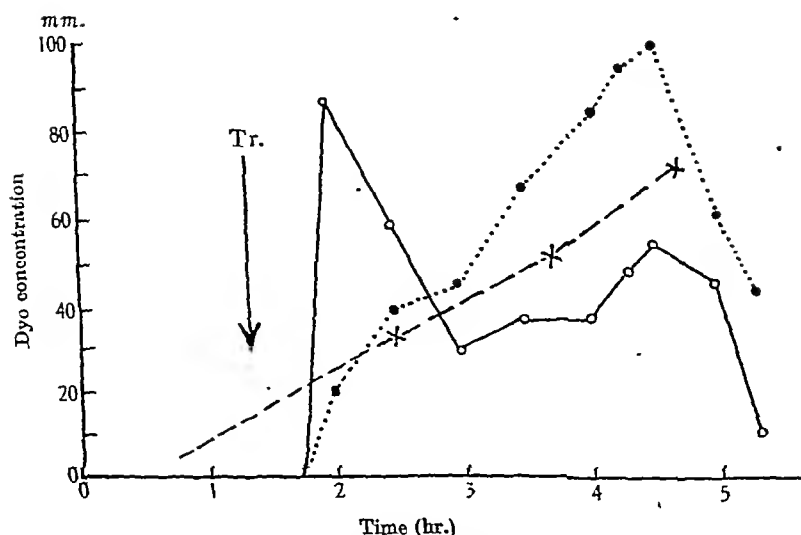
Fig. 1. Dye concentration of the two knee perfusates ½ hr. after trauma; no sympathectomy. Dotted line: concentration of the left, normal, knee perfusate. Solid line: concentration of the traumatized but not denervated right knee perfusate. Interrupted line: blood dye concentration (reduced to 1/100). Arrow: trauma. *Note.* Increased concentration on the traumatized side immediately after trauma, lasting for 1½ hr. and decreasing after this period below the level of the left control side.
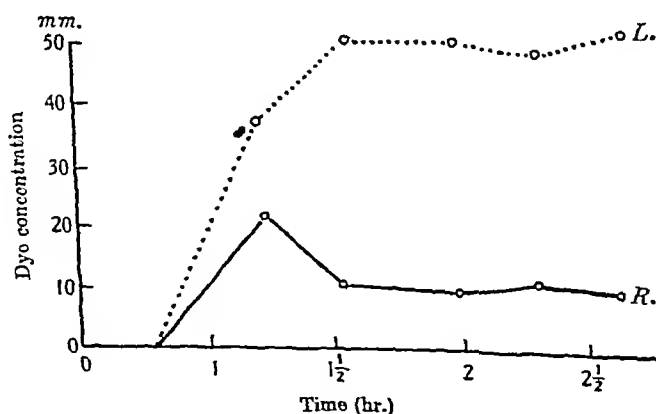
Fig. 2. Dye concentration of both knee perfusates of a dog 3 days after right lumbo-sacral sympathectomy (no trauma on either side). Dotted line: concentration on normal left side. Solid line: concentration on denervated right side. *Note.* The dye concentration on the denervated side is much lower.

The average results of these experiments are compared in Table 1 with those of the earlier controls. In the sympathectomized animals (series *a*), crushing had little effect on the dye concentration of the knee-joint perfusate as compared with the normal limb, either in the 3 hr. immediately following the trauma (8 % increase) or in the later period (5 % increase). In the non-sympathectomized controls, however·(series *b*), crushing resulted in a considerable increase in dye concentration immediately following the trauma (by 163 %) and later in a reduction (to 52 %).

TABLE 1. Average dye concentration of knee perfusates

	During first 3 hr.			After 3 hr.	
Series *a*					
Normal limb (B)	Sympathectomized and traumatized limb (A)	A/B	Normal limb (B)	Sympathectomized and traumatized limb (A)	A/B
89·3	97	1·08	54	57	1·05
Series *b*					
Normal limb (B)	Non-sympathectomized traumatized limb (A)	A/B	Normal limb (B)	Non-sympathectomized traumatized limb (A)	A/B
80	210	2·63	162	85	0·52

These figures show that the increased dye filtration seen during the first 3 hr. after trauma in normal animals is counteracted by the sympathectomy.

The maximum increase in dye concentration in the total perfusate of the crushed side after sympathectomy was 25 %, while the minimum increase in the crushed non-sympathectomized control was 100 %, the maximum being 600 %. There was only one sympathectomized animal in which the dye concentration of the crushed side nearly reached the level of the concentration of the control group; this showed an increase of 80 %. It is noteworthy that in this exceptional case the interval between the sympathectomy and perfusion was the longest in the series, namely, 9 days. It appears that the effect of sympathectomy wears off after a certain period.

Fig. 3 illustrates the dye diffusion from the two knees of a typical case, 3 days after sympathectomy. The dye concentration in the perfusate of the crushed right side was well below the level of that of the normal left side during the first half of the experiment. A constant feature of the present series was the absence of decreased excretion in the second period of the experiments, i.e. 2–5 hr. after the trauma, in contrast to all animals of the non-sympathectomized controls as shown in Fig. 1 and also Table 1. This absence of decrease was apparent in relation both to the excretion of the same side during the first period and to that of the normal control side during the second period.

Another observation made in nearly all animals of the present series was the absence of swelling of the traumatized limb. A non-sympathectomized

leg, after being crushed, swells very considerably, partly due to bleeding, partly due to oedema [Engel & Forrai, 1943]. The average increase in weight of the latter was about 100–150 g. In those four sympathectomized cases in which the weight of the two limbs was measured according to Blalock's technique [1940], the increase of the crushed limb amounted to only 30–50 g.; but it was evident from the macroscopical appearance of the limbs of all other sympathectomized animals that they were much thinner than those of the non-sympathectomized controls. A less developed oedema was noticed also at post-mortem.
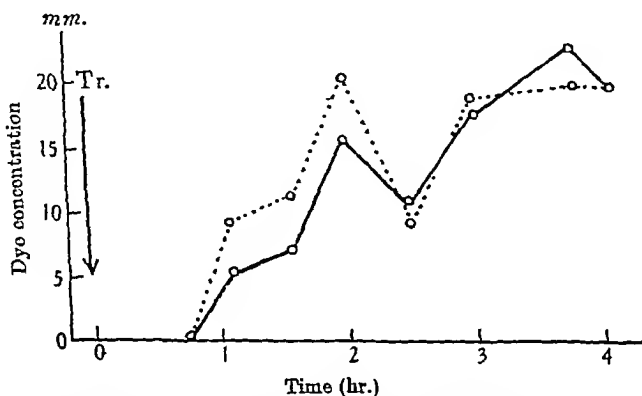
Fig. 3. Dye concentration of the two knee perfusates 3 days after right lumbo-sacral sympathectomy; perfusion started 40 min. after trauma. Dotted line: concentration of normal knee perfusate. Solid line: concentration of traumatized, denervated knee perfusate. Arrow: trauma. *Note.* The excretion in the right knee is lower than that in the left knee, with the exception of a short period. Compare this with reversed relations of Fig. 2.

Both legs crushed after unilateral sympathectomy

The comparison of results given in Table 1, of the effect of trauma on sympathectomized limbs, suffers from the drawback that the comparison was made between different groups of animals. It was therefore considered advisable to examine whether similar results would be obtained if one compared the dye excretion of two crushed limbs in the same animal, one side being sympathectomized as before, the other keeping its normal nerve supply. Three such experiments were performed.

A typical curve of one of the three experiments is shown in Fig. 4. In this animal a right sympathectomy was performed 3 days before both calves were hammered for 5 min. each, with similar intensity, and the two tibiae fractured at their distal end. The fuchsin infusion and the knee perfusion were started immediately after the trauma and carried on for 4 hr. As seen in Fig. 4, the dye concentration in the perfusate from the right sympathectomized side was half that of the normal side. The ratio of the two total perfusates was 1 : 2·1.

This result differs in one respect from the average results given in Table 1. On the basis of those results, one would have expected in the later part of the experiment a reduction in the dye excretion of the traumatized non-sympathectomized leg below that of the traumatized denervated limb. It is possible that this expected reduction of dye excretion in the non-sympathectomized limb was delayed unduly, for the two curves are approaching each other at the end of the experiment. In view of this discrepancy, however, attention should be directed primarily to the effect of sympathectomy on the immediate results of trauma.
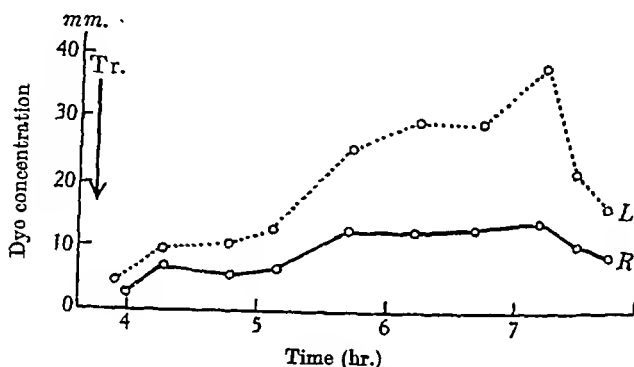
Fig. 4. Dye concentration of the two knee perfusates 3 days after right lumbo-sacral sympath-ectomy. The calves of both legs were traumatized shortly before perfusion. Dotted line: concentration of traumatized, non-denervated left perfusate. Solid line: concentration of traumatized, denervated, right perfusate. Arrow: trauma. *Note.* The dye concentration on the sympathectomized right side is less than half of that of the control side.

In the other two experiments of this series the results were similar: in one the dye concentration on the sympathectomized side was considerably de-creased, the ratio being 1 : 1·5, in the other the concentration was either slightly increased during some period of the experiment, or the two sides were equal; the ratio of the two total perfusates was 1·27 : 1.

DISCUSSION

It has been shown that the rate of filtration through the capillaries is decreased in the area adjacent to trauma and probably in the area of trauma itself, by regional sympathectomy. This decrease in filtration rate was equally manifest, whether the dye filtration of the sympathectomized crushed leg was compared with that of the non-sympathectomized crushed leg of the same animal, or whether the difference in dye concentration between the crushed sympathec-tomized leg and the normal leg of one animal was compared with the difference of concentration between the crushed but non-sympathectomized leg and the normal leg of another animal.

It seems as if one dealt with two superimposed processes: one, produced by the traumatic shock, acting in a positive, filtration-increasing sense; the other, produced by the sympathectomy, acting in a negative, filtration-decreasing sense. The quantitative relation of these two factors explains why the dye excretion was reduced in individual cases to different degrees. If, for instance, the trauma by itself would have increased the excretion by 600% and the sympathectomy only decreased it by 400% the ultimate result would be an increase of 200% compared with the normal side. Had, in the same case, the increasing factor been only 200%, the result would be an absolute decrease by 200%.

It is noteworthy that, in spite of the fact that in the control experiments in which the sympathectomy was performed without trauma only about 75% of the sympathectomies had a decreasing effect on the dye excretion, in the present series there was only one single experiment in which the dye concentration, after the trauma, was increased to the same extent as in the non-sympathectomized animals. Even those experiments in which a slight increase in filtration was recorded on the traumatized side, after sympathectomy, may therefore be considered as positive results, if compared quantitatively with the controls.

It was discussed in two previous papers why the dye excretion has been accepted, though with some reservations, as indicating the capillary permeability. In those two experimental series blood flow was recorded by the thermo-electric method. This was omitted in the present series because the experimental conditions were in many respects similar to those in the two former series, and because our conclusions concerning the relation of filtration rate and permeability of the capillaries to blood flow seemed to be applicable also to the present experiments.

It was shown in our former control experiments that the period of increased dye filtration lasts only 2–5 hr. after trauma, to be followed by a period of decreased dye filtration. This second period never developed after crushing sympathectomized limbs. The reason given for the decreased dye filtration in the control was that the double filtering membrane, consisting of capillary endothelium and synovial membrane, changes into a more composite one by interposition of serum as a third layer. As the sympathectomy, in our present series, reduces the rate of filtration of the capillary endothelium, the exudation of serum through the capillary wall into the intermembranous space and thus the formation of a third layer is also reduced. The hindrances placed in the way of dye filtration will therefore be diminished. There will consequently be no reduction in dye filtration in the second phase of the sympathectomized crushed limb.

The literature on the role of the sympathetic nervous system on shock has been recently reviewed in the excellent monograph of Harkins [1941], and

will therefore be treated only briefly. He came to the conclusion that 'the presence of adrenal medullary overaction with (secondary) overaction of the sympathetic nervous system and peripheral vasoconstriction definitely is a factor in shock. It certainly begins early in the course of shock, but whether it is an initiating, accompanying or perpetuating factor, is difficult to determine.' Neither is it decided, says Harkins, whether the overactivity of the adrenal system is a protective measure of the organism and therefore desirable, or whether attempt should be made to counteract it.

A study on the influence of the sympathetic nervous system on capillary permeability in the literature [Cannon, 1923; Freedman & Kabat, 1940; Freeman, 1933; Freeman, Shaw & Snyder, 1936; Freeman, Freedman & Miller, 1941; Hamlin & Gregerson, 1939] shows that all conclusions are based on secondary manifestations of shock, such as increased haemoglobinemia, and decreased blood volume. It is true that these signs may indicate an increased capillary permeability, but it is known that they do not necessarily do so, and therefore cannot be accepted as conclusive proofs.

The direct evidence that the sympathetic nervous system has an influence on filtration through the capillary wall, and probably capillary permeability in shock, was lacking. The present experiments were meant to fill this gap.

The interesting experiments of Lina Stern will be discussed elsewhere.

SUMMARY

It has been shown by a direct method that it is possible to reduce or prevent, by regional sympathectomy, the locally increased capillary permeability, as manifested by an increased rate of filtration, in traumatic 'shock'.

The effect of the sympathectomy is manifest immediately, as well as several days after operation.

The sympathectomy abolishes the second phase of decreased capillary filtration, characteristic of traumatic shock, which develops 2–5 hr. after trauma [Engel & Forrai, 1943].

These findings are in harmony with, and are direct results of, former experimental evidence showing, first, that sympathectomy reduces capillary permeability in the normal animal, and secondly, that filtration through the capillaries in traumatic 'shock' is locally increased during the first 2–5 hr. after trauma, and decreased after this period.

The therapeutic application of these results in the form of blocking the regional sympathetic chain with novocain during the first phase of increased capillary filtration in traumatic shock will be discussed elsewhere.

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THE BLOOD VOLUME OF NORMAL ANIMALS

By F. C. COURTICE

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The importance of the determination of the circulating blood volume has increased in recent years as a result of a greater demand for knowledge of the phenomena associated with 'shock', produced by various agents such as trauma and burns. The accurate measurement of the blood volume, however, has always involved and still involves many difficulties. The two practicable methods available at present are the carbon monoxide method and the dye method first used by Keith, Rowntree & Geraghty [1915]. Dawson, Evans & Whipple [1920] investigated a large number of dyes with a view to their suitability for blood-volume determination, and were the first investigators to recommend the blue dye, T-1824, which has since been used a great deal for this purpose. It is this dye which has been used in the present series of experiments on animals—rabbits, dogs, goats and horses. Investigations of the problem of shock have been made, but the present paper concerns mainly the results obtained on normal, healthy, unanaesthetized, resting animals.

METHODS

The sample of dye used in these experiments was made by Messrs Eastman, Kodak Ltd., U.S.A. It was shown not to diffuse through a cellophane membrane either in plasma or normal saline [Short, 1943]. It was also shown by means of the ultracentrifuge that it combines with the plasma proteins [Ogston, 1943].

The concentration of dye in the plasma was measured with the aid of a photoelectric cell, the general arrangement of the apparatus being similar to that described by Kennedy & Millikan [1938]. The cell containing the plasma had a volume of approximately 0·6 c.c. with a depth of fluid of 1·5 mm. To estimate the concentration of dye in the plasma, a reading was taken with the sample of plasma free from dye and again with the plasma containing the dye. In this way calibration curves were made using rabbit, dog, goat and horse plasma, the dye concentrations used being 5, 10, 15, 20, 25 and 30 mg./l. These curves were checked at frequent intervals with plasma from the same species of animal. A different curve is obtained if water or saline

is used, so in all cases calibration curves were made with dye in oxalated plasma.

In the determination of the plasma volume, the amount of dye introduced was that calculated to give a concentration of approximately 15 mg./l. in the plasma, that is, about 0·8 mg. of dye per kilogram body weight was used. The dye was used in the following strengths: 0·1 % for rabbits, 0·2 % for dogs and goats and 2·0 % for horses. Blood samples were withdrawn from and dye introduced into the ear veins in rabbits and the jugular veins in dogs, goats and horses. The blood samples were withdrawn from one side of the animal, while the dye was injected into the opposite side. All animals were healthy, at rest and unanaesthetized.

To estimate the blood volume, a sample of blood (about 1·5 c.c. in the rabbit, and 5–10 c.c. in the larger animals) was withdrawn into a dry tube containing potassium oxalate. The dye was then injected into a vein on the opposite side, and 6 min. after the injection of the dye a second sample of blood was withdrawn. The concentration of the dye in the plasma was determined with the photoelectric cell and the plasma volume thus calculated. The cell volume was then determined by means of the haematocrit, and the total blood volume calculated. Ten capillary tubes were spun for 45 min. at 3000 r.p.m., and the mean reading taken as the haematocrit value. Duplicate groups of ten were often done. The calculation for the total blood volume and cell volume assumes that the relation of cells to plasma is constant throughout the blood stream, and that the haematocrit gives a true value for this cell-to-plasma ratio. Gregersen & Schiro [1938] have shown that about 4 % of the dye is trapped between the corpuscles when blood is spun in haematocrit tubes for 30 min. at 3000 r.p.m., and it is probable that the haematocrit value may vary in samples of blood taken from different parts of the body. Neither of the above assumptions is, therefore, necessarily strictly correct, but the errors cannot be very large.

RESULTS

Rate of disappearance of dye from the plasma. One of the greatest difficulties involved in the dye method is the choice of a time after injection of the dye at which to withdraw the second sample of blood. At this time mixing of the dye must be complete and no dye must have disappeared from the circulation. A time of 6 min. was chosen by Kennedy & Millikan and this has been adhered to here. The amount of dye which has disappeared from the circulation during this time is difficult to determine accurately, and the rate of disappearance varies in individual animals.

In Table 1 the rates of disappearance of the dye in rabbits, goats and dogs can be seen to vary in individual animals. The mean disappearance rates are plotted in Fig. 1, and the curves extended back to zero. From these experiments it seems probable that complete mixing had taken place by 6 min., as

TABLE 1. Rate of disappearance of T-1824 from the blood stream of normal, unanaesthetized animals. Figures are the dye concentrations of the plasma in mg./l.

	Group (i) Min. after dye injection						Group (ii) Min. after dye injection		
	6	15	30	45	60		6	12	18
Rabbits	15·7	14·5	13·6	13·0	12·3		23·3	21·9	20·8
	16·7	15·0	14·4	13·6	13·2		13·2	13·1	12·8
	13·5	12·5	11·5	11·4	11·1		18·3	17·1	15·9
	16·0	15·0	14·1	12·8	11·8		10·1	9·8	9·5
	13·8	12·7	12·1	11·0	10·5		14·0	13·3	12·7
	13·0	12·1	11·2	10·5	10·0		10·8	10·0	9·6
Mean	14·8	13·6	12·8	12·1	11·5	Mean	15·0	·14·2	13·6
Goats	10·5	9·8	9·0	8·4	8·0		12·5	11·5	10·8
	9·8	9·5	8·7	8·5	8·1		13·5	12·7	11·8
	14·2	12·7	11·1	10·6	10·0		11·7	10·6	9·4
	15·2	13·4	12·0	11·2	10·9		12·7	11·5	11·2
	13·2	11·8	10·9	10·5	9·4		18·0	17·5	16·8
Mean	12·6	11·4	10·3	9·8	9·3		11·7	11·2	11·0
							17·0	15·6	14·5
							15·5	14·3	13·3
						Mean	14·1	13·1	12·4
Dogs	13·6	12·6	11·7	—	10·7		16·1	15·0	14·5
	14·3	13·3	12·6	—	12·0		18·3	17·8	17·7
	15·6	14·3	13·3	—	12·4	Mean	17·2	16·4	16·1
	12·7	12·0	11·1	—	11·1				
	14·1	13·2	13·0	—	12·5				
Mean	14·1	13·1	12·3	—	11·7				

in all cases the concentration of dye was highest in the 6 min. sample. The trend of the curves for goats and rabbits is very much the same, whereas in dogs the fall in the curve is much slower in the second half-hour. In order to ascertain how much dye diffuses from the circulation in the first 6 min., the graphs in Fig. 1 were extended backwards to zero. By this means the value obtained at 6 min. is only 94% of the theoretical concentration at zero in rabbits and dogs and 93% in goats. This is a mean figure only. Individual variations can be seen in Table 1.

Amount of dye re-entering the blood stream via the lymph. The calculation of the amount of dye disappearing from the blood stream in the first 6 min. will be true only if no dye is re-entering the blood via the lymphatics during the time that the disappearance curve is being determined; it is reasonably certain that none enters in the first 6 min. Ferrebee, Leigh & Berliner [1941] have measured the amount of dye in the plasma, thoracic duct lymph and cervical duct lymph after injection of T-1824 intravenously in dogs. They found that dye appeared in the thoracic duct and cervical duct lymph within the first hour, and that in one dog 7·5% of the injected dye was collected from the thoracic duct in 2 hr.

In the present series of experiments the amount of dye appearing in the thoracic duct lymph has been determined in six dogs and four goats. The dogs were anaesthetized with nembutal intraperitoneally and the goats with

sodium barbitone intravenously. The thoracic duct was cannulated, and the lymph collected in 6 min. samples for 12 min. before dye injection and for 1 hr. after dye injection. Blood samples were collected from a cannulated carotid artery. The dye in the plasma was estimated as already described. The variations in the opalescence of the thoracic duct lymph made it impossible to measure accurately the dye concentration in the lymph samples
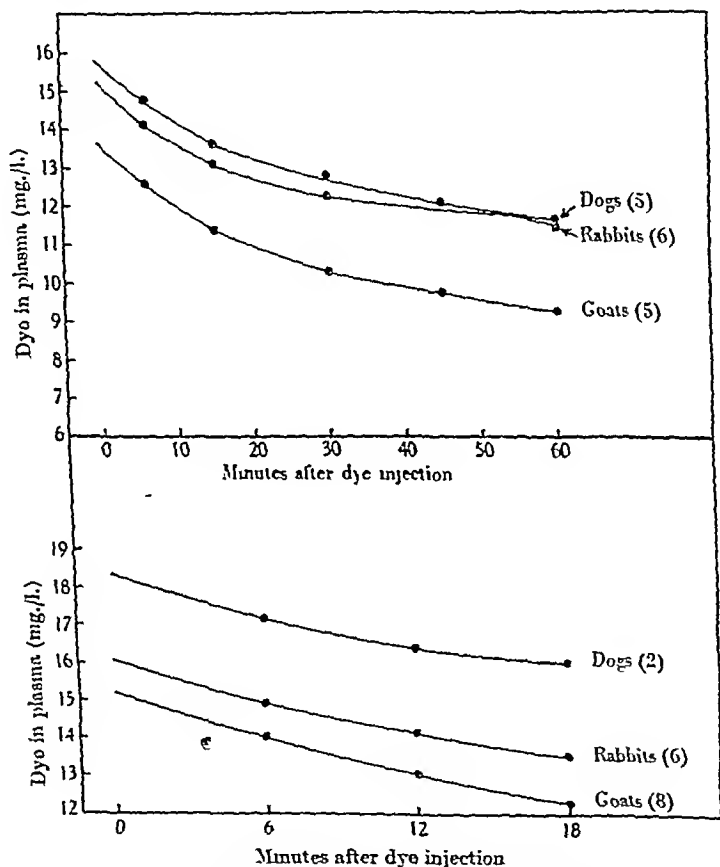
Fig. 1. Mean rates of disappearance of T-1824 from the plasma of normal, unanaesthetized dogs, rabbits and goats.

directly. In these samples, therefore, the dye was always extracted with butyl alcohol by the method described by Harington, Pochin & Squire [1940]. In this way the dye concentration in the lymph could be accurately determined.

The mean results for the six dogs and four goats are shown in Fig. 2, while the individual results are given in Tables 2 and 3. These results show that dye always appears in the thoracic duct lymph about 15 min. after injection and gradually increases during the hour. In the dog the mean concentration

of dye in the thoracic duct lymph of six animals at the end of 1 hr. w‹
4·2 mg./l. compared with 13·1 mg./l. in the plasma, that is, about 32% of th
plasma level. In the goat the mean concentration of dye in the thoracic duc
lymph of four animals at the end of 1 hr. was 2·2 mg./l. compared wit
11·4 mg./l. in the plasma, that is, about 19% of the plasma level. Althoug
the concentration of the dye in the thoracic duct lymph reaches a fairly hig‹
level, the actual amount of dye which appears in this lymph is very small
amounting in 1 hr. to approximately 0·5% of the amount injected (Table 3)
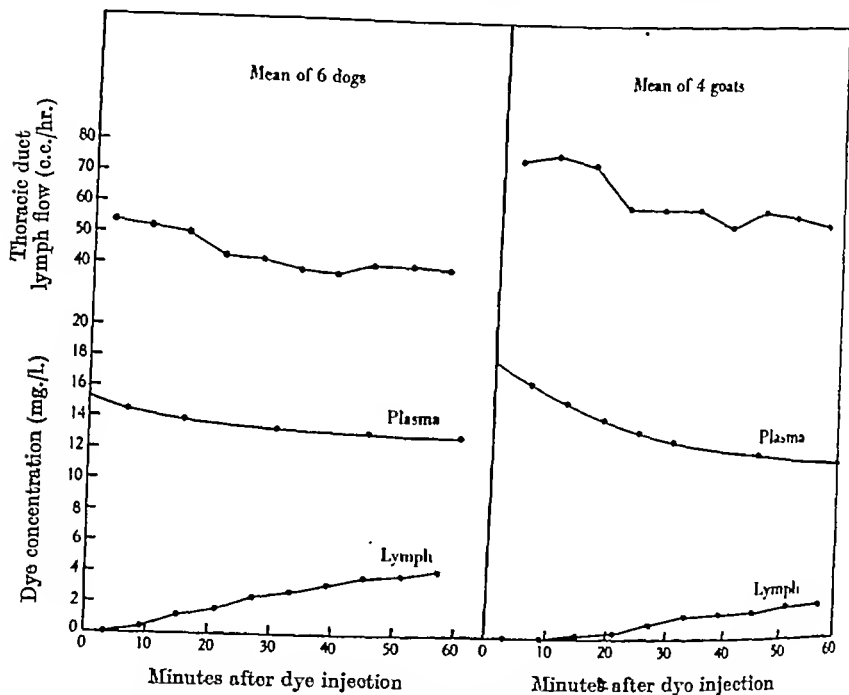
Fig. 2. Thoracic duct lymph flow and concentrations of T-1824 in plasma
and thoracic duct lymph after intravenous injection of the dye.

Even though the concentration and total amount of dye in the thoracic duct
lymph in the dog is slightly greater on the average than in the goat, the rate
of disappearance of dye from the plasma is much less in the dog. The rate of
fall of the plasma concentration in the anaesthetized dog is less than in the
unanaesthetized, whereas in the goat and rabbit it is about the same whether
unanaesthetized or anaesthetized. The reason for these differences is difficult
to explain. According to Gibson & Evans [1937] the dye is removed from the
blood stream principally by phagocytosis in the reticulo-endothelial system.
Possibly in the goat and rabbit this system removes the dye more quickly
than in the dog.

TABLE 2. *Rate of disappearance of T-1824 from the blood plasma and amount of dye appearing in thoracic duct lymph in anaesthetized dogs and goats*

Dogs

Thoracic duct lymph — Volume, c.c.

Time after injecting dye min.	1	2	3	4	5	6
0– 6	3.2	7.0	0.2	7.5	3.7	4.0
7–12	3.4	7.0	0.3	7.1	3.7	3.5
13–18	3.4	7.2	0.0	0.5	3.7	2.5
19–24	2.8	0.3	0.6	4.1	3.6	2.5
25–30	2.8	0.7	0.8	3.1	2.6	2.8
31–36	2.7	0.3	0.5	2.5	2.0	2.5
37–42	2.7	5.0	0.0	3.0	2.0	2.1
43–48	2.7	0.5	0.3	4.0	2.0	1.0
49–54	3.2	5.0	0.0	3.1	2.5	2.5
55–60	3.0	0.0	0.0	4.5	1.8	2.4

Thoracic duct lymph — Dye concentration, mg./l.

Time after injecting dye min.	1	2	3	4	5	6
0– 6	0.2	0.0	0.0	0.0	0.0	0.0
7–12	0.8	0.1	0.2	0.0	0.3	1.0
13–18	1.5	0.8	1.5	0.4	1.0	1.8
19–24	2.0	1.5	1.5	0.0	1.3	2.3
25–30	3.2	2.7	1.4	1.0	2.0	3.7
31–36	3.5	2.5	1.8	2.1	2.0	4.2
37–42	4.3	2.8	2.5	2.1	2.5	5.0
43–48	5.0	2.8	2.0	2.7	3.0	5.0
49–54	5.0	3.1	3.4	2.0	3.0	5.3
55–60	5.0	3.2	4.0	2.0	3.0	6.2

Blood plasma — Dye concentration, mg./l.

Min. after dye	1	2	3	4	5	6
0	16.2	16.2	13.3	12.7	14.3	14.0
15	15.2	15.0	13.3	12.4	13.0	13.6
30	14.3	15.4	13.3	11.0	13.3	13.3
45	13.0	15.8	13.3	11.7	13.0	13.2
60	13.0	15.6	13.1	11.7	12.3	13.3

Goats

Thoracic duct lymph — Volume, c.c.

Time after injecting dye min.	1	2	3	4
0– 6	12.0	8.3	4.1	5.0
7–12	13.0	7.5	4.5	6.4
13–18	12.0	4.0	5.5	4.4
19–24	12.0	4.0	5.2	3.0
25–30	11.0	5.1	5.8	3.5
31–36	10.5	4.5	6.1	3.3
37–42	9.5	4.5	5.2	2.0
43–48	10.5	4.3	7.0	2.8
49–54	9.5	4.3	6.4	3.1
55–60	8.5	4.0	5.9	3.4

Thoracic duct lymph — Dye concentration, mg./l.

Time after injecting dye min.	1	2	3	4
0– 6	0.0	0.0	0.0	0.0
7–12	0.0	0.0	0.0	0.0
13–18	0.0	0.2	0.7	0.3
19–24	0.2	0.2	1.0	0.3
25–30	1.7	0.3	2.0	0.2
31–36	2.8	0.3	2.5	0.3
37–42	3.2	0.3	2.7	0.7
43–48	2.4	1.0	2.7	1.0
49–54	3.0	1.3	3.2	1.0
55–60	3.0	1.3	3.5	

Blood plasma — Dye concentration, mg./l.

Min. after dye	1	2	3	4
0	11.8	19.8	19.0	10.6
12	11.5	17.5	17.8	15.2
18	11.2	15.8	16.2	14.3
24	11.2	14.9	15.3	13.5
30	10.6	13.8	14.0	12.0
45	9.8	12.0	14.0	12.0
60	9.4	12.0	13.0	11.4

TABLE 3. Amount of injected dye appearing in thoracic duct lymph

Dogs	Weight kg.	Amount of dye injected mg.	Total dye in lymph in 1 hr. mg.	% of injected dye in lymph in 1 hr.
1	15·5	14	0·096	0·69
2	23·0	20	0·118	0·59
3	24·6	20	0·122	0·61
4	23·0	20	0·065	0·33
5	13·6	10	0·048	0·48
6	13·6	10	0·086	0·86
			Mean	0·59
Goats				
1	31·0	24	0·160	0·67
2	9·0	8	0·020	0·25
3	16·4	16	0·110	0·69
4	14·5	12	0·011	0·09
			Mean	0·43

The amounts of lymph from the cervical and right lymph ducts have been measured in many experiments for a different purpose and have been so small compared with the flow from the thoracic duct that they have been ignored here. In only one dog of the series in Table 2 was the cervical lymph collected, and in 1 hr. no dye appeared.

It can be concluded from these experiments that the amount of dye returning to the blood stream via the lymphatics during the first hour after dye injection is very small and may be neglected in the anaesthetized animal. The fall in the plasma disappearance curve can, therefore, be assumed to be due to an outward passage of dye from the circulation, not complicated by a considerable re-entry of dye into the circulation. Thus, by extending the curves backwards to zero, a reasonable indication of the loss of dye in the first 6 min. after injection is obtained.

In the estimations of the blood volume to be given below, only one sample of blood was taken after the dye was injected, viz. at 6 min. It would probably be more accurate to take several samples after dye injection and determine the disappearance curve in each case. This is inadvisable when using small animals (rabbits) if the blood volume has to be estimated daily for a week or more, and also when dealing with unanaesthetized animals suffering from shock, the investigation of which was proposed. Therefore, in all experiments to be described, it has been assumed that complete mixing has taken place in 6 min. after injection of the dye, and that in this time 6 % of the dye injected has disappeared from the circulation.

Diffusion of the dye into the corpuscles. That the dye does not diffuse into the corpuscles is shown by the following experiment: 2 c.c. of 2·0 % dye and 248 c.c. of blood were mixed in a 250 c.c. flask. The concentration of the dye in the plasma was estimated at varying intervals after mixing, the flask being shaken frequently in the meantime. The corpuscular and total blood volumes were calculated from the haematocrit reading. The results are shown in Table 4.

TABLE 4

	Corpuscles c.c.	Plasma c.c.	Total c.c.
Actual volume	68	182	250
Estimated volume 10 min. after mixing	67	176	243
,, 45 ,,	67	178	245
,, 180 ,,	67	176	243

The estimation of the dye concentration in the plasma was made by the direct method, i.e. by estimating the dye in the plasma without extraction. In some instances this method was compared with the concentration estimated by extraction of the dye with butyl alcohol [Harington et al. 1940]. The difficulty of the latter method in small animals is the large amount of blood necessary. In the estimations on the rabbit all quantities were one-fifth those described. The results of the two methods in a small series of rabbits, goats and horses are shown in Table 5. In the rabbit and goat the plasma is

TABLE 5

		Direct method			Extraction method		
		Plasma vol. c.c.	Corpuscle vol. c.c.	Total vol. c.c.	Plasma vol. c.c.	Corpuscle vol. c.c.	Total vol. c.c.
(a) Rabbits		108	53	161	108	53	161
		196	64	260	185	63	248
		91	53	144	100	58	158
	Mean	132	57	189	131	58	189
(b) Goats		2180	580	2760	2180	580	2760
		3150	1370	4520	3070	1330	4400
	Mean	2665	975	3640	2625	950	3580
		l.	l.	l.	l.	l.	l.
(c) Horses		28·60	12·40	41·00	28·40	12·40	40·80
		24·85	10·65	35·50	25·00	10·70	35·70
	Mean	26·73	11·53	38·25	26·70	11·55	38·25

fairly clear and colourless after being centrifuged for about 1 hr. at 3000 r.p.m. In the horse, however, the plasma has a definite yellowish colour. The results in Table 5 suggest that in normal animals the amount of yellow pigment does not affect the method. If, however, the plasma is deeply coloured with bile as in certain cases of hepatitis, the direct method is not accurate, the colour obtained after injection of the dye being a dirty grey instead of blue. In these cases the bile pigments can be removed by Harington's extraction method and the dye estimated in the butyl alcohol.

The accuracy of the blood-volume estimations is difficult to determine. The method is accurate *in vitro*, but *in vivo* several difficulties have already been mentioned. However, fairly constant results on the same animal can be obtained on different days. Table 6 shows the blood-volume determinations in four rabbits on five successive days and Table 7 the blood volume on two

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successive days in fifty rabbits, eight dogs and eight goats. Daily variations occur, but these may be partly due to an actual change in the blood volume and not necessarily to an error in its determination.

TABLE 6

Rabbit no.	Blood volume c.c., days				
	1	2	3	4	5
1016	177	187	200	203	198
1017	164	160	172	157	181
1018	196	198	195	194	192
1019	192	188	203	208	198
Mean	182	183	192	190	192

TABLE 7

	Blood volume in c.c.					
	Days		Days		Days	
	1	2	1	2	1	2
Rabbits	196	194	182	188	171	172
	221	216	165	166	116	117
	219	223	165	156	150	165
	168	166	181	208	139	142
	220	202	194	167	208	186
	236	198	149	147	154	166
	215	235	141	142	228	240
	167	181	154	166	183	200
	276	274	170	167	233	248
	200	230	134	140	158	157
	228	217	136	136	165	152
	203	212	170	194	159	137
	221	219	180	181	131	175
	190	197	135	138	202	192
	186	187	171	150	223	240
	193	194	165	161	124	126
	188	194	127	126 Mean	180	182
Dogs	2405	2580	658	714	1110	1200
	2425	2540	1155	1183	1540	1615
	611	686	1030	921 Mean	1367	1430
Goats	3050	2960	2610	2370	2040	2060
	1600	1840	1505	1400	2350	2440
	2095	2080	1485	1455 Mean	2092	2076

The blood volume of normal rabbits, goats, dogs and horses. In the course of other experiments, the blood volume has been estimated in the following normal, unanaesthetized animals: 60 rabbits, 30 goats, 29 dogs (mongrels), 4 greyhounds (highly trained) and 2 horses. The relation between the blood volume and body weight in the groups of rabbits, goats and dogs is shown in Figs. 3–5. The weights of individual animals in these groups varied considerably, but in all groups the blood volume varied directly as the body weight.

In Table 8 are the mean values for plasma volume, corpuscle volume and total blood volume per kilogram body weight and the haemoglobin percentage
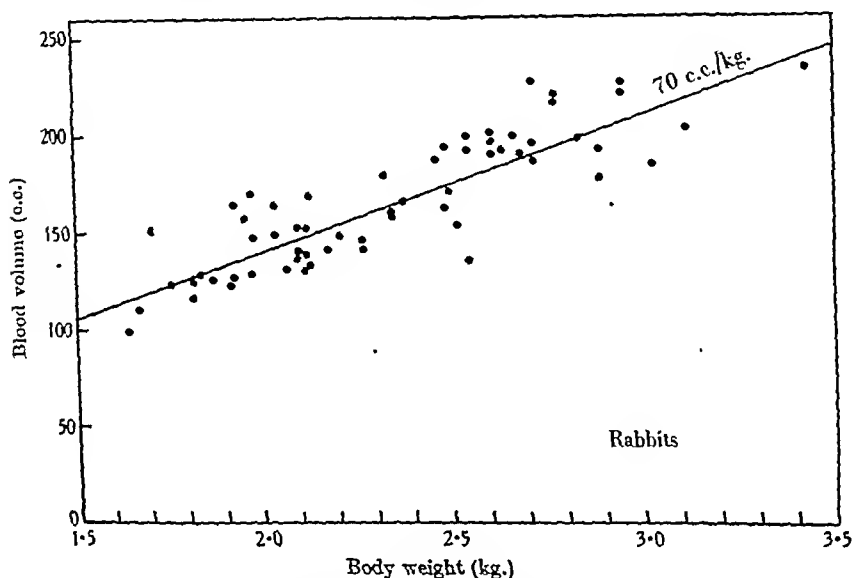
Fig. 3. The relation between blood volume and body weight in 60 rabbits.
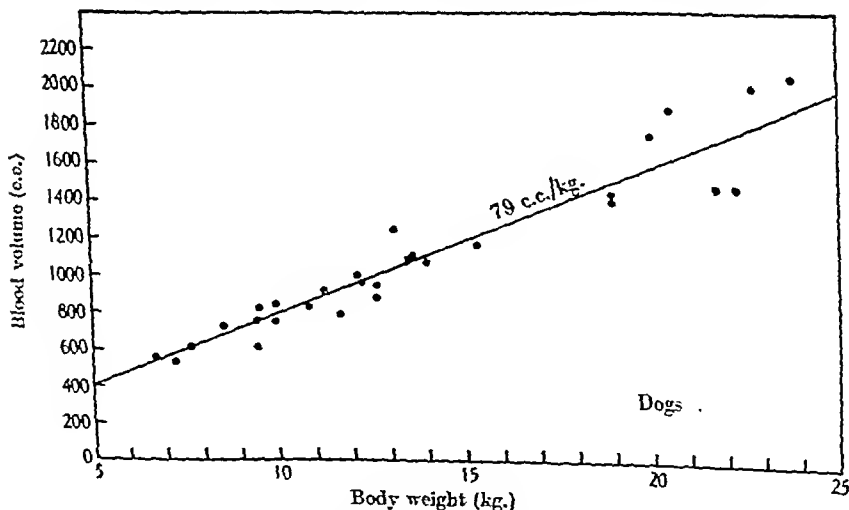
Fig. 4. The relation between blood volume and body weight in 29 dogs.

(Haldane) of each of the groups of animals investigated. The blood volume per kilogram body weight is fairly constant in all groups of animals except in the case of the greyhounds. The greyhounds were highly trained and consisted mainly of bone, blood and muscle. The blood volume seems, therefore,

to be greater in highly muscular animals. The plasma volume, however, is constant in all these groups of animals. It is the cell volume which is so greatly increased in the greyhounds.

Fig. 5. The relation between blood volume and body weight in 30 goats.

It is interesting to compare the relation between the surface area and the blood volume in the different animals. The surface area was calculated from the following formulae: rabbits, $S = 0.125 \sqrt[3]{W^2}$; dogs, goats and horses,

TABLE 8

	Haemoglobin %	Plasma vol. c.c./kg.	Corpuscle vol. c.c./kg.	Total vol. c.c./kg.
Rabbits (60)	68	50	20	70
Goats (30)	68	53	17	70
Horses (2)	—	51	21	72
Dogs, mongrel (29)	88	54	25	79
Greyhounds (4)	133	54	60	114

$S = 0.107 \sqrt[3]{W^2}$, where S is the surface area in square metres and W the body weight in kilograms. In Table 9 are shown the relations between the blood volume and the surface area and the blood volume and the body weight. In these four different species, varying greatly in size, the blood volume per kilogram body weight is fairly constant, whereas the blood volume per square

TABLE 9

	Surface area sq. m.	Body weight kg.	Blood vol. c.c./sq. m.	Blood vol. c.c./kg.
Rabbits (60)	0·222	2·38	759	70
Dogs (29)	0·625	14·07	1780	79
Goats (30)	1·11	32·4	2070	70
Horses (2)	6·76	504·0	5325	72

metre surface area increases as the size of the animal increases and its relative surface area decreases. This can also be well seen in the group of goats where the individual size of the animals varies greatly. Table 10 shows how, as the animal increases in size, the blood volume per square metre surface area increases, whereas the relation to body weight is constant. The blood volume, therefore, depends upon the bulk of the animal tissue, especially muscle, and not upon the rate of metabolism which varies with the surface area.

TABLE 10

Goat no.	Surface area sq. m.	Weight kg.	Blood vol. c.c.	Blood vol. c.c./sq. m.	Blood vol. c.c./kg.
643	0·66	15·5	1050	1591	68
214	0·69	16·4	1218	1760	74
213	0·74	18·2	1200	1623	66
641	0·76	19·0	1285	1691	68
133	0·77	19·1	1255	1630	66
239	0·79	20·0	1640	2075	82
240	0·80	20·4	1592	1990	78
206	0·82	20·9	1485	1810	71
205	0·83	21·4	1505	1815	70
121	0·87	22·7	1592	1830	70
207	0·95	25·9	1720	1810	66
132	1·00	28·2	1910	1910	68
203	1·00	28·2	2095	2095	74
223	1·02	29·6	2040	2000	69
204	1·03	30·0	2370	2300	79
208	1·12	33·6	2443	2180	73
224	1·12	33·6	2350	2100	70
209	1·13	34·1	2443	2165	72
210	1·17	36·4	2595	2220	71
120	1·21	37·3	2960	2445	79
257	1·21	37·7	2820	2350	75
255	1·22	38·6	2470	2025	64
251	1·24	39·5	2245	1810	57
254	1·27	40·8	2885	2275	71
108	1·36	44·6	3055	2245	69
256	1·36	44·6	3000	2200	67
107	1·42	47·8	3520	2480	74
212	1·50	52·3	3540	2360	68
252	1·63	59·6	4005	2460	67
211	1·74	65·9	4250	2440	65

Blood volume in abnormal animals. The results so far give an indication of the blood volume of normal animals only. If the method is to be of practical value, it must also hold in cases of 'shock' where the capillaries, either in a local area or maybe generally, are damaged and are thereby rendered more permeable to plasma proteins. In such cases the peripheral circulation has also partially failed, so that mixing of the dye in the plasma may not be so

to be greater in highly muscular animals. The plasma volume, however, is constant in all these groups of animals. It is the cell volume which is so greatly increased in the greyhounds.

Fig. 5. The relation between blood volume and body weight in 30 goats.

It is interesting to compare the relation between the surface area and the blood volume in the different animals. The surface area was calculated from the following formulae: rabbits, $S = 0 \cdot 125 \sqrt[3]{W^2}$; dogs, goats and horses,

TABLE 8

	Haemoglobin %	Plasma vol. o.o./kg.	Corpuscle vol. c.o./kg.	Total vol. c.c /kg.
Rabbits (60)	68	50	20	70
Goats (30)	68	53	17	70
Horses (2)	—	51	21	72
Dogs, mongrel (29)	88	54	25	79
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224	1·12	33·6	2350	2100	70
209	1·13	34·1	2443	2165	72
210	1·17	36·4	2595	2220	71
120	1·21	37·3	2960	2445	79
257	1·21	37·7	2820	2350	75
255	1·22	38·6	2470	2025	64
251	1·24	39·5	2245	1810	57
254	1·27	40·8	2885	2275	71
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256	1·36	44·6	3000	2200	67
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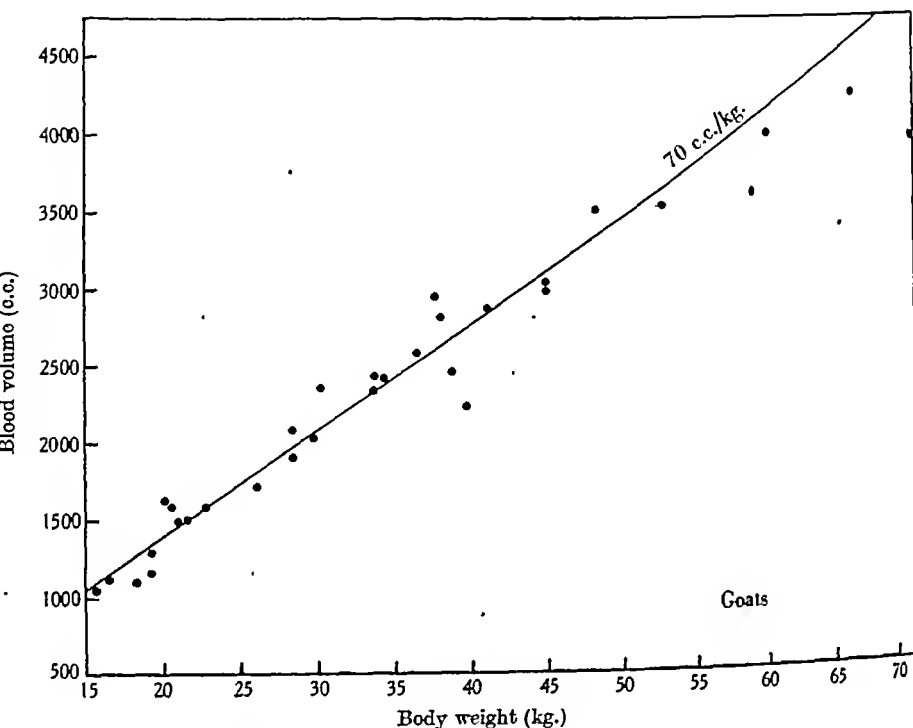

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be measured by the method described. In Table 12, figures are given for the blood volume of four goats before and 16 hr. after exposure to a lung irritant which caused severe pulmonary oedema and haemoconcentration. In all cases the plasma volume has very greatly decreased, corresponding fairly closely with the rise in the haemoglobin percentage and the haematocrit value. In the first two cases the corpuscle volume has remained fairly constant, whereas in the last two cases the corpuscle volume has increased. A rise in the corpuscle

TABLE 12

		Hb %	Haematocrit	Plasma vol. c.c.	Corpuscle vol. c.c.	Total vol. c.c.
(1)	Before	96	31·6	1635	755	2390
	16 hr. after	129	38·6	1185	745	1930
(2)	Before	66	20·6	3380	880	4260
	16 hr. after	88	28·9	2240	910	3150
(3)	Before	64	22·9	1930	570	2500
	19 hr. after	125	41·9	980	705	1685
(4)	Before	52	17·1	2180	450	2630
	19 hr. after	90	32·4	1225	585	1810

volume as in these last two cases has often been noted in cases of severe haemoconcentration due to plasma loss, especially in dogs. Whether this is due to a true increase in corpuscle volume or due to an error has not been ascertained. The spleen may play some part in a true increase. Reasons for the likelihood of errors in such circumstances have already been discussed.

DISCUSSION

The chief difficulties involved in this method of measuring blood volume have already been discussed. The results show that by taking only one sample after dye injection fairly constant and reliable figures are obtained in normal animals. The animal used mostly by other investigators has been the dog. Gibson, Keeley & Pijoan [1938] estimated the blood volume of fifty normal dogs, using the dye T-1824. They found a plasma volume of 41·2–51·7 c.c./kg. and a total blood volume of 84·0–97·3 c.c./kg. These plasma volumes are slightly lower than those in the present series of dogs, but the cell volume is higher. These authors give a summary of blood-volume estimations in dogs by other investigators using various methods. The results obtained varied from 7·6 to 11·9% of the body weight. Recently, Bonnycastle & Cleghorn [1942] have estimated the blood volume of 106 normal dogs using T-1824, and found a plasma volume of 31·8–64·6 c.c./kg. and a total volume of 60–107·5 c.c./kg. The variations seen in the results of different authors may be caused by the different times at which the blood sample is taken after dye injection. Welsch [1905], using the Welcker method in one dog, found a blood volume of 7·5% of the body weight, and he quotes earlier authors, who used this method, as obtaining mean figures of 7·7% of the body weight. The mean figure of 79 c.c./kg. in dogs in the present series agrees closely with these figures.

rapid as in the normal animal. Even though the capillaries are more permeable in shock, the outward flow of plasma compared with the total plasma volume is not so rapid that the dye disappears from the circulation at a much altered rate from normal in a short time. In the case of acute pulmonary oedema produced by a lung irritant, the amount of fluid that passes into the lungs in 6 min. after dye injection is not very great. The rate of disappearance of dye was determined in three normal rabbits and four normal goats, and again next day after they had been exposed to a lung irritant which caused in all cases massive pulmonary oedema. All rabbits were suffering from severe respiratory distress and died about 1 hr. after the experiment, while the goats all showed considerable haemoconcentration, signs of shock and respiratory distress. The disappearance of the dye from the blood stream in these experiments is shown in Table 11. There is not much difference in the rate of

TABLE 11

	Normal					16 hr. after lung irritant			
			Dye in plasma mg./l. (min.)					Dye in plasma mg./l. (min.)	
Goat no.	Hb %	Haematocrit	6	12	18	Hb %	Haematocrit	6	12	18
251	96	31·6	18·0	17·5	16·8	129	38·6	24·7	23·2	22·7
252	66	20·6	11·7	11·2	11·0	88	28·9	17·5	16·8	16·4
254	60	23·5	12·5	11·5	10·8	78	26·0	15·6	14·5	13·7
255	52	17·1	13·5	12·7	11·8	90	32·4	18·7	17·6	15·9
Mean	69	23·2	13·9	13·2	12·6	96	31·5	19·1	18·0	17·2

	Normal				After lung irritant			
	Dye in plasma, mg./l. (min.)				Dye in plasma, mg./l. (min.)			
Rabbit no.	6	15	30	60	6	15	30	60
630	16·7	15·0	14·4	13·2	15·3	14·1	13·5	11·0
631	13·5	12·5	11·5	11·1	14·1	13·2	12·7	10·3
632	16·0	15·0	14·1	11·8	17·7	17·0	15·0	12·7
Mean	15·4	14·2	13·3	12·0	15·7	14·8	13·7	11·3

disappearance during a short time in the normal and shocked animals. Thus although plasma may be passing out of the damaged capillaries, the amount that does so in a short time should not introduce a very large error. If the blood volume is changing rapidly as after transfusion or haemorrhage, larger errors may be involved, but no data are available in these circumstances.

An error of greater significance may be introduced by slower mixing due to peripheral circulatory failure or to variations in the degree of haemoconcentration in different parts of the blood stream. Another error may be caused by haemolysis. In animals suffering from shock with severe haemoconcentration and peripheral circulatory collapse, it is difficult to prevent haemolysis in the sample of blood taken. This is especially so in the dog. If haemolysis is more than slight, large errors will be introduced in spite of the filter.

Only four examples of the blood volume in shock will be given here to show that in spite of many difficulties, a fairly accurate idea of the plasma loss can

be measured by the method described. In Table 12, figures are given for the blood volume of four goats before and 16 hr. after exposure to a lung irritant which caused severe pulmonary oedema and haemoconcentration. In all cases the plasma volume has very greatly decreased, corresponding fairly closely with the rise in the haemoglobin percentage and the haematocrit value. In the first two cases the corpuscle volume has remained fairly constant, whereas in the last two cases the corpuscle volume has increased. A rise in the corpuscle

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volume as in these last two cases has often been noted in cases of severe haemoconcentration due to plasma loss, especially in dogs. Whether this is due to a true increase in corpuscle volume or due to an error has not been ascertained. The spleen may play some part in a true increase. Reasons for the likelihood of errors in such circumstances have already been discussed.

DISCUSSION

The chief difficulties involved in this method of measuring blood volume have already been discussed. The results show that by taking only one sample after dye injection fairly constant and reliable figures are obtained *in normal animals*. The animal used mostly by other investigators has been the dog. Gibson, Keeley & Pijoan [1938] estimated the blood volume of fifty normal dogs, using the dye T-1824. They found a plasma volume of 41·2–51·7 c.c./kg. and a total blood volume of 84·0–97·3 c.c./kg. These plasma volumes are slightly lower than those in the present series of dogs, but the cell volume is higher. These authors give a summary of blood-volume estimations in dogs by other investigators using various methods. The results obtained varied from 7·6 to 11·9 % of the body weight. Recently, Bonnycastle & Cleghorn [1942] have estimated the blood volume of 106 normal dogs using T-1824, and found a plasma volume of 31·8–64·6 c.c./kg. and a total volume of 60–107·5 c.c./kg. The variations seen in the results of different authors may be caused by the different times at which the blood sample is taken after dye injection. Welsch [1905], using the Welcker method in one dog, found a blood volume of 7·5 % of the body weight, and he quotes earlier authors, who used this method, as obtaining mean figures of 7·7 % of the body weight. The mean figure of 79 c.c./kg. in dogs in the present series agrees closely with these figures.

Boycott [1912] found by bleeding that the blood volume of the rabbit was only 45 c.c./kg. This is very different from 70 c.c./kg. found in this series by the use of T-1824. Why the figures for blood volume obtained by early workers using the Welcker method should agree with figures obtained by the dye method in dogs and not rabbits is not understood.

Harington *et al.* [1940] in a small series of human subjects suggest that the blood volume varies as the surface area. Their series, however, is rather small and the difference in size in the individuals is not great enough to come to a definite conclusion. In the whole series of animals reported here, the blood volume has been proportional to the body weight, and not to the surface area. It seems reasonable to expect this. In the smaller animal with a relatively larger surface area and therefore a greater metabolic rate, the circulation rate and not the amount of blood is increased.

In the few examples given of the estimations of blood volume in conditions of abnormal capillary permeability, the changes in plasma volume can be fairly accurately determined. By taking only one sample of blood after dye injection instead of a series, it is possible to determine the blood volume daily for a period of a fortnight or longer even in small animals, with what appear to be accurate results. The degree of accuracy *in vivo* is impossible to determine as it is unlikely that the blood volume of any animal remains exactly the same from day to day.

SUMMARY

The blood volume of a series of normal, healthy, unanaesthetized rabbits, dogs, goats and horses has been estimated with the blue dye T-1824. The mean values for 60 rabbits, 29 dogs, 30 goats and 2 horses are respectively: plasma volume, 50, 54, 53 and 51 c.c./kg. body weight and blood volume 70, 79, 70 and 72 c.c./kg. The blood volume of four highly trained greyhounds was much higher due to a higher cell volume.

The blood volume in these animals, varying greatly in size, is proportional to the body weight and not to the surface area.

The amount of dye re-entering the blood stream via the thoracic duct in 1 hr. after dye injection is very small. The mean value in six dogs was 0.59% and in four goats 0.43% of the amount of dye injected. The rate of disappearance of dye from the blood stream during the first hour is therefore not affected by a considerable, and possibly varying, re-entry of dye via the lymphatics.

A few examples of the blood volume in 'shock' are given, which show that the method gives reliable results in conditions of abnormal capillary permeability.

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DIFFERENTIATION IN THE ABSORPTION OF OLIVE OIL AND OLEIC ACID IN THE RAT

By A. C. FRAZER,* *From the Physiology Department, St Mary's Hospital Medical School, London, and the Pharmacology Department, University of Birmingham*

In studies of fat absorption various triglycerides have been fed to animals, but the absorption of ingested fatty acids has never been systematically investigated. According to the current hypothesis fat is completely hydrolysed before it can be absorbed, and resynthesis of neutral fat occurs in the intestinal cell with the aid of intermediate phosphorylation and the adrenal cortical hormone [Verzár & McDougall, 1936]. The reconstituted triglyceride passes by the lacteals to the thoracic duct and so to the systemic circulation. No significant quantity of fat is thought to pass by the portal vein nor is the absorption of unhydrolysed fat considered a possibility. If this hypothesis is correct, one would expect fatty acid and glycerol to behave during and after absorption in a manner similar to neutral fat. The object of this paper is to show that such a correlation does not exist, and to offer a possible explanation of the differences observed.

METHODS

Animals. Throughout the experiments described, adult male rats from our own stock were used.

Materials. The fats used were olive oil and redistilled oleic acid. When oleic acid was administered glycerol was given as well in amounts equivalent to the corresponding triglyceride. Except where stated elsewhere, the fatty materials fed to the animals were stained prior to use with Sudan IV to make a 0·1% solution of the stain in oil. By the use of fat or fatty acid labelled with Sudan IV, its passage to the depots or the liver can be determined.

Feeding. The animals were fed by stomach tube without anaesthesia when detailed measurement of the quantity of olive oil administered was required. This method was not found suitable for long-term experiments. In these the fatty material was fed mixed with the food and the amount consumed was checked against faecal analyses.

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Analyses. Estimation of fat administered and of the amount remaining in the intestine at the end of the experimental period was made by the gravimetric modification of Bloor's method previously described by Elkes, Frazer & Stewart [1939]. In some instances faecal analyses were used over a period determined by charcoal administration.

Chylomicrographs. Investigation of the passage of fat along the various pathways was made by simple observation in the case of lacteals and the chylomicrograph for changes in systemic or portal blood fat. This method for studying blood fat, originally described by Gage & Fish [1924], was fully investigated and standardized by Frazer & Stewart [1939]. Samples of blood for chylomicrographs were withdrawn from the tail vein for the systemic blood or direct from the portal vein.

Operation. For the simultaneous portal and systemic chylomicrographs, the animals were kept anaesthetized with urethane and the portal specimens obtained direct from the portal vein through a small abdominal incision.

Histology. Histological examination of the intestinal cells was made in frozen sections prepared by standard technique.

RESULTS

Amount of fatty material absorbed

Using thirty-six rats no significant difference was detected between the percentage absorption of olive oil or of equivalent amounts of oleic acid and glycerol. In all cases 80–90 % of the fatty material reaching the intestine was absorbed and quantities up to 1 g. were taken up from the intestinal lumen during the experimental period. Similar results were obtained with faecal analyses in long-term experiments. Animals fed on fat or fatty acid over periods of several weeks showed normal growth and weight curves and no increase of fatty material in the faeces.

Appearance of the intestinal cells

Seventy rats were used in these experiments and sections were examined from animals killed at hourly intervals. After feeding with neutral fat, a characteristic picture is seen. Above the bile duct there is very little evidence of fat absorption. In the lower part of the duodenum and upper part of the jejunum the intestinal cells are filled with large Sudan-staining globules of fat which can be seen from 1 hr. onwards (Plate 1A). Only in that part of the duodenum, which is just below the bile duct, does the picture closely resemble that obtained with oleic acid.

When oleic acid and glycerol are fed on the other hand, the cells throughout the intestine are filled with a finely granular, fatty, Sudan-staining material (Plate 1B). These fine particles do not appear to unite to form the large globules which are seen after feeding with neutral fat.

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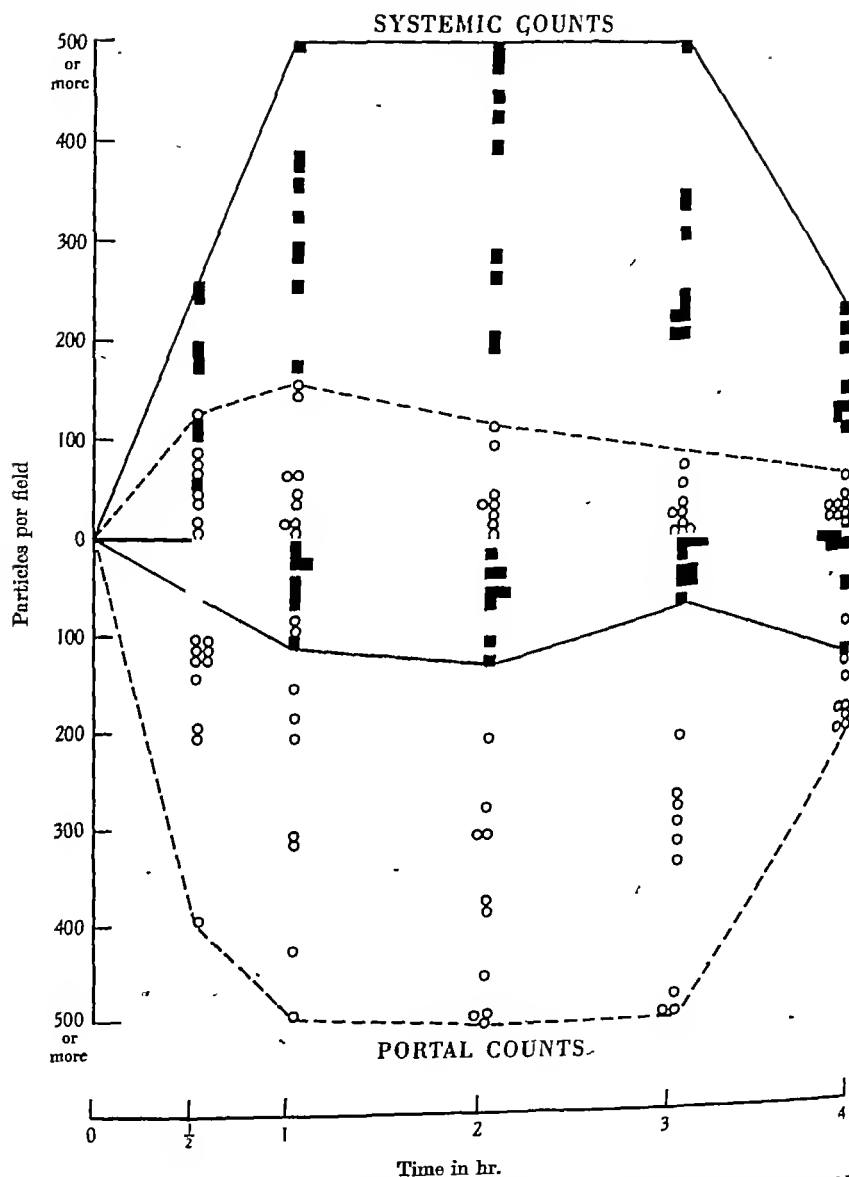
Text-fig. 1. Simultaneous portal and systemic particle counts. Specimens prepared from blood samples taken simultaneously from the portal and systemio systems in rats following the ingestion of neutral fat or fatty acid and glycerol. The squares are from counts after feeding triglyceride, while the circles are from those following fatty. acid. The continuous lines indicate the maximum counts after triglyceride and the dotted lines the maxima after an equivalent amount of fatty acid.
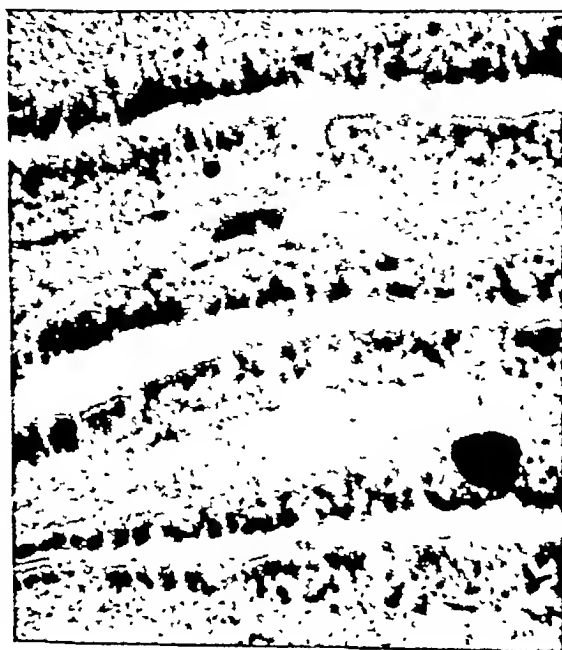
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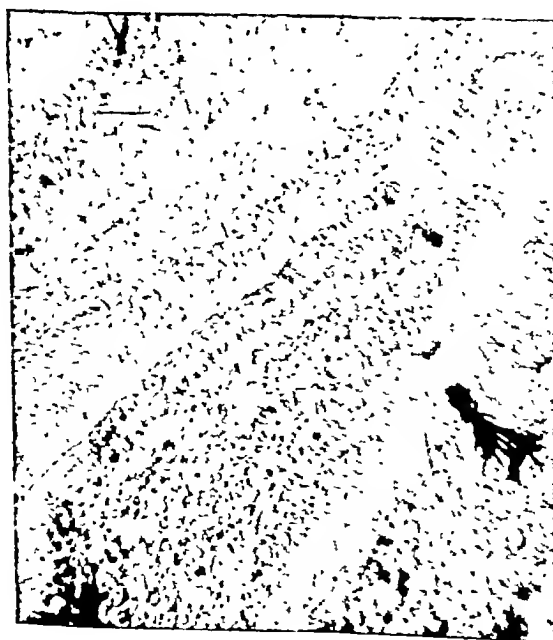
B

A

B

Pathways of absorption

In these experiments twenty rats were used. After feeding with neutral fat, sections of the villi show large quantities of fat in the areolar tissue corium and in the central lacteal. The lacteals on macroscopic examination appear milky. Simultaneous portal and systemic particle counts show a large increase of particles in the systemic blood but only a small rise in the portal blood (Text-fig. 1).

If an equivalent amount of oleic acid and glycerol is administered the areolar tissue and lacteals are relatively free from fatty material, and the lymphatic vessels in the root of the mesentery do not appear milky. Simultaneous portal and systemic counts reveal that the portal blood has a marked increase of particles, whereas the systemic blood shows very little increase (Text-fig. 1).

Destination of absorbed fat

The observations in this section are based on the examination of thirty-six rats. If groups of rats are given 2 c.c. of Sudanized olive oil mixed with their ordinary food, for a period of 1 week, the fat depots are found to be deeply stained. No such staining is apparent if equivalent amounts of stained oleic acid and glycerol are fed.

In the demonstration of liver fat, a surplus of fatty material must not be given as this leads to considerable accumulation of fat in the liver due to flooding of the fat depots. The 2 c.c. or more of fatty material, which is commonly used in absorption experiments in rats, is much in excess of the normal amounts ingested. If 1 c.c. of Sudanized olive oil is fed to a rat and the animal is killed after 5 hr. there is a relatively small amount of Sudan-stained fatty material visible in the liver (Plate 2A). If an exactly similar quantity of Sudanized oleic acid is fed, large quantities of stained material are found in the liver (Plate 2B). It should be noted that in these liver sections no other fat stain is used apart from Sudan IV which is in the original fatty material fed to the animal.

DISCUSSION

It is apparent from these observations that there are fundamental differences in the behaviour of neutral fat as compared with fatty acid and glycerol both during and after absorption. From analyses of the residual contents of the intestine or of the faeces, it is unquestionable that comparable amounts of the fat or fatty acid ingested disappear from the lumen. It must be assumed that these amounts have been absorbed, and indeed it can be demonstrated that absorption has occurred in each case. The picture obtained, however, with neutral fat is quite different from that seen with fatty acid. In the intestinal cell neutral fat gives large globules of oil; this appearance is very similar to that described by Jeker [1936] 6 hr. after ingestion of oil. Fatty acid gives a fine granular deposition, which stains more brownly with Sudan

and which resembles more closely in appearance that described by Jeker [1936] as occurring some 30 min. after ingestion of olive oil. In our experience this type of fatty acid-containing cell is found only in that part of the intestine which is just below the pancreatic duct after feeding with olive oil, but over a wide area if fatty acid itself is administered. It appears as though the fatty acid is in a much finer state of division than neutral fat when it is contained within the intestinal cell. Verzár & McDougall [1936] have cited Jeker's findings as evidence for resynthesis of neutral fat from fatty acid. There is, however, nothing to show that such a conversion actually occurs, and the two differing pictures are easily obtained by taking sections from two different parts of the intestinal tract, or by taking the sections at varying time intervals after the administration of olive oil. Sections of intestinal cells taken within an hour or so of ingestion of neutral fat show mainly a fatty acid picture—if they are taken 6 hr. later the cells show the characteristic appearance of neutral fat absorption.

From the study of the pathways leading from the intestine, the neutral fat globules seem to pass into the lacteals giving rise to a milky appearance of the intestinal lymphatics, thence the fat-laden chyle empties by way of the thoracic duct into the systemic blood. Within an hour of ingestion of neutral fat, there is always a striking increase of fat in the systemic blood. Fatty acid does not pass into the lymphatic system, but into the portal capillaries. It is perhaps significant that in other parts of the body particulate dyes when injected tend to pass into the lymphatics while water-soluble substances go direct into the blood stream. Fatty acid is certainly in a very fine state of division, and according to some authorities [Verzár & Kúthy, 1930] it is absorbed as a water-soluble complex with bile acid. The fatty acid, like other water-soluble absorbed substances, passes by the portal vein, and thus, after ingestion of fatty acid, there is no great increase of fat in the systemic blood. Using labelled fat the destination of absorbed fat can be determined. Gage & Fish [1924] found that stained fat fed to animals caused staining of the fat depots, but no investigators, so far as we are aware, have used stained fatty acid in following out the destination of absorbed fatty material. As would be expected if stained neutral fat is fed, its destination is found to be the main fat depots. Heavy staining is found not only in the subcutaneous depots but also in the omental and perirenal areas. Stained fatty acid fed over a period of 10 days results in no staining of these areas. On the other hand, feeding with stained, neutral fat, provided that it is not excessive in amount, does not cause much deposition of stained fat in the liver, but the feeding of stained fatty acid results in a striking accumulation of Sudanized fatty material. It may be suggested that labelling with Sudan is not a reliable method, but we have tried to upset the Sudan labelling of our samples by hydrolysis and other methods, and we have never succeeded in separating

the Sudan stain from the oily fraction. The temporary disappearance of Sudan coloration when the fat is very finely dispersed can be easily explained and readily demonstrated with Sudanized oil. It does not have any significant bearing upon the interpretation of our experiments.

It is not possible to correlate these findings with the lipolytic hypothesis. An explanation of our observations, which is also in accord with the experimental results of other workers, is afforded by the partition hypothesis [Frazer,
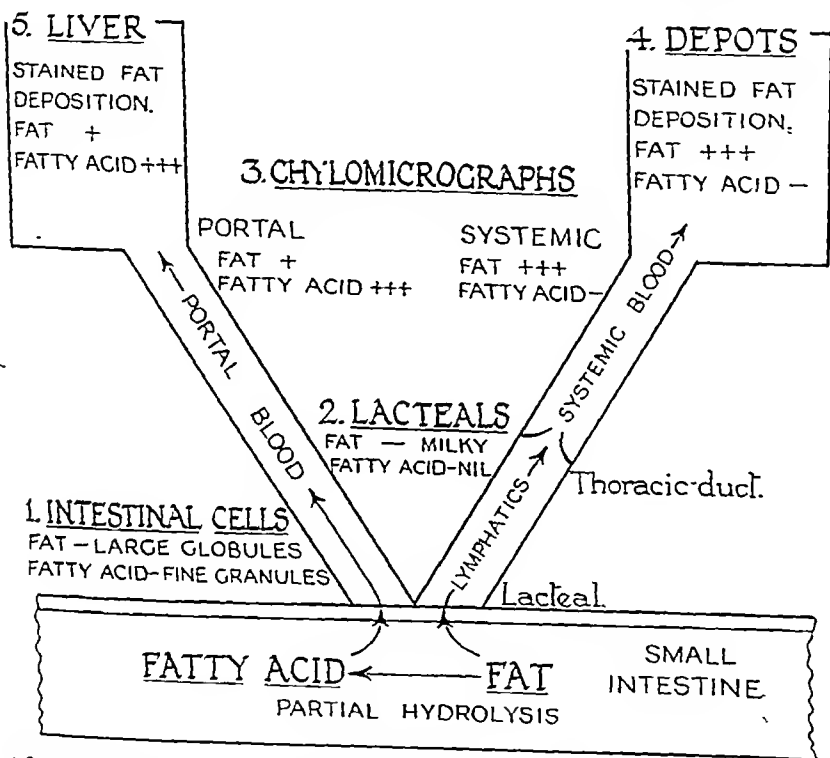
Text-fig. 2. The partition hypothesis of fat absorption. The diagram shows the partition of fat and fatty acid by partial hydrolysis in the intestine, and it indicates the main evidence upon which the hypothesis is based.

1938]. According to this view (Text-fig. 2) lipolysis is only partial, and hydrolysis of the triglyceride molecule is not regarded as an essential preliminary to its absorption. The split and unsplit fractions of neutral fat are absorbed by different mechanisms into the intestinal cell, where they give rise to different histological pictures, and from which they pass by different routes to different destinations. Fatty acid passes by the portal vein to the liver, while neutral fat goes by the lymphatic route to the systemic blood and thence to the depots to be stored for future use. The degree of lipolysis is, thus, a determining factor in the immediate fate of absorbed fat.

SUMMARY

1. Neutral fat absorption gives rise to large globules in the intestinal cell, whereas fatty acid absorption shows a fine brown granular deposit.

2. Neutral fat absorption is accompanied by milky lacteals; not to fatty acid absorption.

3. Neutral fat absorption gives a systemic lipaemia, but little change in the portal blood. Fatty acid causes a marked portal lipaemia with little change in the systemic blood.

4. Neutral fat can be traced to the fat depots, and provided it is administered in moderate doses, it fails to give marked deposition in the liver. Fatty acid, on the other hand, does not appear in the fat depots, but it gives rise to a marked deposition in the liver.

5. These findings cannot be correlated with the current hypothesis of complete lipolysis prior to absorption.

6. The partition hypothesis of fat absorption is put forward as a possible explanation of the observations reported.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

A. Section from the small intestine of the rat 6 hr. after feeding with olive oil. The fat is present in the intestinal cells in large Sudan-staining globules. Magnification × 300. B. Section from the small intestine of the rat 6 hr. after feeding with fatty acid and glycerol: the fatty material is in the form of fine brown granules in striking contrast to the fat in A. Magnification × 300.

PLATE 2

A. A section of liver from the rat killed 4 hr. after feeding with 1 c.c. Sudanized olive oil. A small amount of stained fatty material is found. Magnification × 300. B. A section of liver from a rat killed 4 hr. after feeding with 1 c.c. Sudanized oleic acid with an equivalent amount of glycerol. Marked accumulation of stained fatty material is found throughout the liver. Magnification × 300.

THE MICROPHONIC EFFECT OF TELEOST LABYRINTHS AND ITS BIOLOGICAL SIGNIFICANCE

By Y. ZOTTERMAN, *From Karolinska Institutet,*
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In the present paper a series of experiments is described dealing with electrical effects led off from the labyrinth of the pike, *Esox lucius*, and the burbot, *Lota vulgaris*. The sacculus of these fishes produces a microphonic effect. as originally found by Adrian. Craik & Sturdy [1938] for the pike and the eel as well as for reptiles and amphibians. The microphonic effect of the inner ear first observed by Wever & Bray [1930] in mammals seems thus not to be limited to species which possess a fully-developed cochlea.

The Wever & Bray effect has been subjected to many studies concerning its origin. Several ideas have been put forward. Thus it has been attributed in turn to the nerve fibres, to the hair cells of the organs of Corti, and to Reissner's membrane or any other polarized membrane of the cochlea. The fact that a very similar effect can be led off from the sacculus of these fishes offers special means for solving. in principle, the problem concerning the structures which are responsible for the phenomenon. In surviving preparations of the pike and burbot the boundaries of the macular tissue can be seen by the naked eye so that the sacculus of these fishes, particularly of the latter, offers especially good facilities for exploring the site of generation of the microphonic effect.

Besides this phenomenon the impulses elicited from the posterior semicircular canal have been studied by leading off the action potentials from the nerve strand running from the posterior cupola.

TECHNIQUE AND PROCEDURE

After decapitation. the head was divided by a sagittal section in the midline. The labyrinths of both sides were now open to free inspection. That part of the eighth nerve which spreads radially to the macula sacculi can easily be dissected free from the surrounding tissue (Fig. 1. *rs*). The electrodes were

placed on isolated strands of this nerve. The nerve running to the posterior ampulla (Fig. 1, *rap*) was similarly isolated and in order to avoid interference from the lagena the branch running to its macula (Fig. 1, *rl*) was severed. The nerves had to be handled with the greatest care in order to avoid mechanical damage. Repeated failures to obtain working preparations in the beginning seemed to be due to mechanical damage. The electrodes used were of the common Ag-AgCl type, and the electrical response was recorded by means of a resistance-capacity coupled amplifier and a cathode-ray oscillograph. The origin of the sacculus effect was explored with the aid of a micro-electrode and a micro-manipulator kindly put at my disposal by Prof. R. Granit.
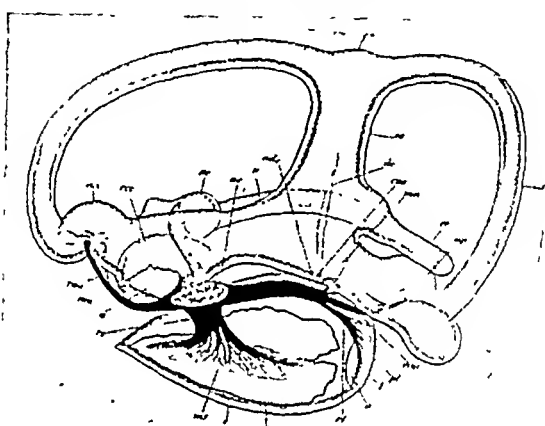
Fig. 1. The labyrinth of *Esox lucius*, after G. Retzius [1884]. *ac*, the acoustic nerve; *rs*, nerve branch running to the macula sacculi, *ms*; *rap*, nerve branch to the posterior ampulla; *rl*, branch to lagena.

In order to study the impulses produced in the posterior cupola the preparation was fixed on a cork disk in a gyroscopic device of the kind described by Ross [1936] in his study of the frog's labyrinth. This apparatus made it possible to study the effects of angular displacements in all planes.

RESULTS

The sacculus effect

In the preparations used, the electrical response from the sacculus and that from the nerve strands running to the macula sacculi can be studied separately. With one electrode on the nerve and the other on the sacculus the two effects obtained in response to a tuning-fork of 60 cyc./sec. in contact with the operation table can be seen combined as in Fig. 3A. There is a sinusoidal oscillation which follows the frequency of the fork. On these waves a number of spikes can be seen occurring fairly regularly at the end of the rising phase
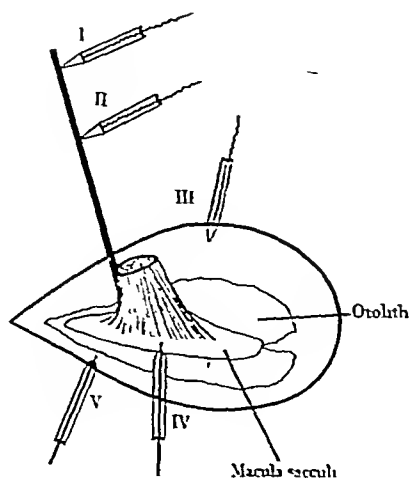
Fig. 2. Diagram showing arrangement of electrodes in experiments upon the sacculus. I, II and III, Ag-AgCl electrodes; I and II, placed upon a nerve strand running to the macula sacculi; III, electrode placed upon the sacculus. IV and V, micro-electrodes; IV, placed upon the macula; V, placed outside the macular region.
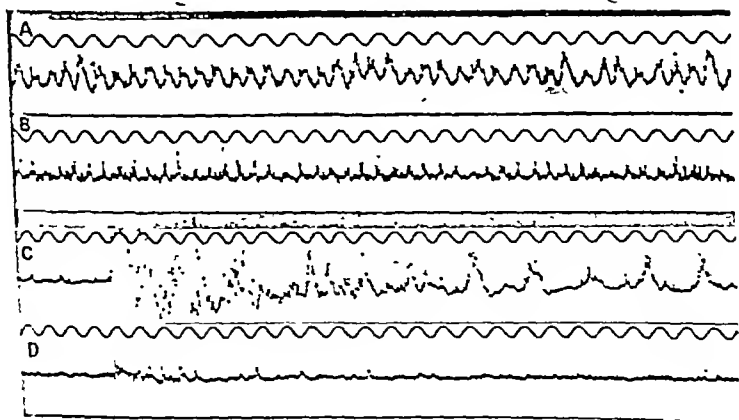
Fig. 3. Electrical effects from the sacculus nerves; *Esox lucius*. The two upper records show the response to a tuning-fork of 60 cyc. sec. In A one electrode was placed on the nerve and the other on the sacculus. In B both electrodes were placed on the nerve. The lower records show the response to a light tap on the preparation table with corresponding electrode arrangements. Time marker, 50 cyc. sec.

of each wave. When both electrodes are placed on the nerve as in Fig. 3B, the sinusoidal current nearly vanishes, while the spikes remain, appearing regularly as single spikes or brief volleys of spikes at the frequency of the stimulus. In Fig. 3 C, D the effect of a light tap on the preparation table with corresponding electrode arrangements can be seen.

The origin of the sacculus effect

As already stated the large sacculus of the burbot offers special facilities for exploring the site of the microphonic phenomenon. For this purpose the effect was picked up by a micro-electrode, which was shifted along the surface
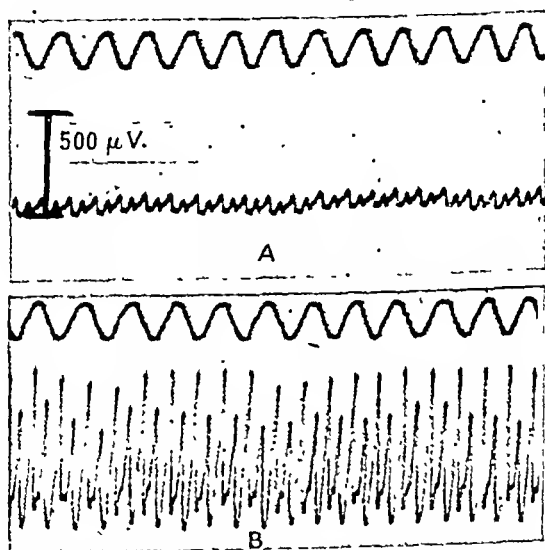
Fig. 4. Records of the microphonic effect of the sacculus of *Lota vulgaris*. The effect was picked up by a micro-electrode placed A outside, B inside the macular region.

of the sacculus. As the electrode approached the macular region the effect increased, and when it passed the border of the macula there was regularly a very strong increase in the amplitude, which often exceeded by ten times or more the value obtained by leading off from a point outside the macular region. The difference can be seen in Fig. 4.

This observation strongly suggests that the microphonic effect is generated in the macula itself. It thus raises the much debated question whether the microphonic phenomenon is purely an incidental feature or whether it serves a definite physiological purpose.

When using strong stimuli the effect led off from the sacculus may attain values of several millivolts, which indicates that the intrinsic potential most likely reaches values of hundreds of millivolts or more. Thus even fairly weak

stimuli must set up potentials within the macula, which are of such a magnitude that they can hardly leave the nerve fibres within the macula unaffected. The records A and C of Fig. 3 show how the impulses are set up at the end of each rising phase in accordance with this view. It suggests that the microphonic effect, obviously originating within the macula, could be regarded as a true generator potential, which is set up within the receptor cells and which in its turn excites the nerve fibres concerned. In their recent experimental analyses of the effect of electric potentials applied to afferent nerve fibres, Granit & Skoglund [1943] have shown how it is possible to reproduce the phenomena observed in afferent fibres in response to natural stimuli by applying potentials of various gradients and strengths to the nerve fibre itself. The microphonic effect from the sacculus which might be called the macular effect could then be regarded as a generator potential similar to the retinal potential. Thus it seems very likely that the cochlear phenomenon serves a similar purpose. There are so far no facts which seem to be contradictory to this view.

The response from the posterior ampulla

The effect of accelerated rotation about all axes of the labyrinth was observed in eight surviving preparations of the pike and in five preparations of the burbot. In both species an outburst of impulses was elicited by angular displacements when the ampulla was leading. Fig. 5 shows a record
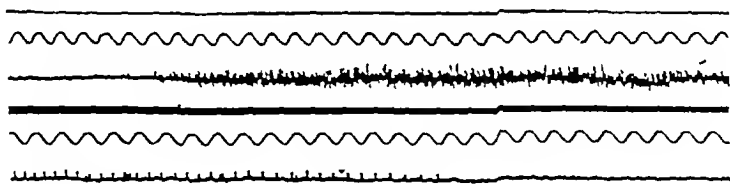
Fig. 5. Action potentials led off from the nerve strand running to the right posterior ampulla of *Esox lucius* in response to anticlockwise rotation about a transverse axis. The records show the beginning and the end of the response.

of the action potentials from the nerve of the right posterior ampulla of the burbot in response to an anticlockwise rotation about a transverse axis. When it is rotated in the opposite direction no immediate effect can be seen, but there is a quite definite after-discharge when the movement is stopped. This after-discharge is generally well pronounced in fresh preparations but diminishes gradually as the preparation ages, and it vanishes earlier than the direct response to ampulla-leading displacements.

In most preparations there has been very little spontaneous activity. As seen in Fig. 5 there is no activity when the preparation is at rest. In other preparations, as shown in Fig. 6, where a spontaneous activity was observed, this activity was not inhibited when the ampulla was exposed to relative

movements of the endolymph in the direction of the ampulla. In this respect the results obtained from the ampullar nerves of the pike and the burbot differ from the findings by Löwenstein & Sand.[1936, 1941] in the elasmobranch labyrinth.
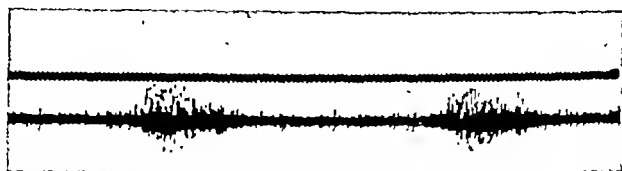
Fig. 6. Record from the nerve to the posterior ampulla of *Lota vulgaris* in response to rotary movements.

SUMMARY

1. The microphonic effect of the sacculus has been studied in surviving preparations of the labyrinth of the pike and the burbot. Evidence has been produced which suggests that the saccular effect is generated within the macula, and the biological significance of the phenomenon considered as a generator potential discharging the nerve fibres is briefly discussed.

2. The electric response from the nerve of the posterior ampulla is of phasic nature occurring in response to angular displacements in one direction only. Rotation in the opposite direction does not produce any immediate effect in these fishes but only an after-discharge when the movement is stopped.

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SOME EXPERIMENTS ON THE POSSIBLE RELATIONSHIP BETWEEN VITAMIN C AND CALCIFICATION

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Although it seems well established that adequate amounts of vitamin C are essential for the laying down of the organic matrix of bone, opinions differ as to whether it is also necessary for the production of bone salt.

Salter & Aub [1931] showed that a scorbutic diet prevented deposition of calcium in bones but did not show that vitamin C allowed calcification to take place (because vitamin C was not yet available for experimental purposes). Boyle [1938] and Boyle, Bessey & Howe [1940] state that the process of calcification continues in the teeth of guinea-pigs even when they have been for some time on a scorbutic diet. Wolbach & Bessey [1942] state that it is a generally accepted fact that vitamin C plays no part in the process of calcification. A more detailed review of the literature concerning the effect of scurvy on calcium absorption and metabolism has already been given by Bourne [1942c]. The only reference in the literature to the inhibiting effect of a scorbutic diet on the deposition of calcium in bone appears to be that of Salter & Aub. It was necessary therefore to investigate this problem further.

METHODS AND RESULTS

Forty-seven guinea-pigs were used for this work and the problem was approached in the following five ways:

(1) Confirmation of the fact that a scorbutic diet inhibits calcification in bones and investigation of whether pure vitamin C added to a scorbutic diet permits calcification to take place normally.

(2) Investigation of whether the calcification of bony trabeculae which are formed by regenerating bone is affected by scurvy.

(3) Investigation of the effect of a scorbutic diet on the calcification of the bony trabeculae of costo-chondral junctions.

(4) Investigation of whether substances normally present with vitamin C in citrus fruits, i.e. citrate and vitamin P (citrin), have any effect on bone matrix formation or calcification.

(5) Further confirmation of the fact that alkaline phosphatase activity of bones is reduced in scurvy.

(1) Calcification of bones was demonstrated by Salter & Aub [1931] by the injection of sodium alizarin sulphonate into animals. The rationale of the technique has been explained by Cameron [1930]. When calcification is going on, the newly deposited bone salt is, by this method, stained pink. The less bone salt deposited the less pink colour is produced on the bone.

In the first experiment a dye kindly provided by Prof. J. C. Brash of Edinburgh was used. It was 1:2:5:8-tetrahydroxyanthraquinone (Alizarin Bordeaux). Alizarin itself is dihydroxyanthraquinone. Twelve guinea-pigs were placed on a scorbutic diet (a mash of bran, wholemeal flour, and cod-liver oil with rat cake *ad lib.*; see Bourne [1942*b*]), and were given daily doses of vitamin C by subcutaneous injection, the vitamin being injected to ensure that each animal got its exact dose. Each set of two animals received daily 5, 2·5, 1·25, 0·5 and 0·25 mg. of the vitamin. The remaining two animals received no injections of vitamin C.

A quantity of the above-mentioned dye was added to the mash each day, but the guinea-pigs as a result refused to eat the mash. It was evident also that, even if they were persuaded to eat it, those guinea-pigs which ate more than the others would get more of the dye and would therefore have their bones more intensely stained than those which ate less. Therefore after another 2 days, that is, at the end of the first week, each guinea-pig was given daily, with a pipette, 1 g. of dye suspended in 10 c.c. distilled water. This was continued for a week. The animals were then killed, the femora dissected out, carefully cleaned of muscle and fibre and examined while fresh.

The bones of animals receiving 2·5 and 5 mg. vitamin C stained the best, but the latter were no better than the former. The bones of the animals receiving 1·25, 0·5 and 0·25 mg. stained with equal intensity, but worse than those which received 2·5 and 5·0 mg.: at the same time they were better than the bones of the completely scorbutic animals. This experiment suggested then that vitamin C does play a part in the process of laying down bone salt, but it does not show how far the deposition of bone salt is simply dependent upon the availability of the necessary matrix.

(2*a*) For this experiment three guinea-pigs were placed on a scorbutic diet. In this and subsequent experiments the scorbutic diet was the same as that described by Bourne [1942*b*]. After 13 days a 1 mm. hole was bored aseptically in each femur by a method described by Bourne [1942*a*]. Feeding with dye was begun the day after the operation, i.e. after the animals had been on a scorbutic diet for 2 weeks. Of these animals, one was given 2 mg. vitamin C

daily by injection for the whole of the experiment (3 weeks), another was given 0·5 mg. vitamin C, and the third was given no supplement of the vitamin. One week after the operation the animals were killed, the femora dissected out and examined while fresh. The staining of the tissue filling the hole was best in the animal which received 2 mg. vitamin C. There was slight staining in the animal which had received 0·5 mg. of the vitamin, and there was no staining at all in the hole in the completely scorbutic animal.

(2*b*) One would expect lack of calcification of the repair tissue in vitamin C deficiency, because calcium salts are not deposited until osteoid trabeculae are formed, and it has been shown by Bourne [1942*b*] that these trabeculae do not form or are only formed in small amounts in scorbutic animals.

In the experiment now to be described, therefore, the animals were operated on (in this case a hole about 5 mm. in diameter was bored as usual in each femur) while they were still enjoying an ample diet, and immediately after the operation they were placed on a scorbutic diet. It is usually accepted that guinea-pigs take about 7 days to use up their body reserves of vitamin C [Giroud, 1938], and therefore even in a guinea-pig on a completely scorbutic diet there was probably enough vitamin C in the body reserves during the first 7 days to permit the development of osteoid trabeculae, but it was thought that there might be insufficient amounts of the vitamin (if it does play a part in calcification) to permit the deposition of normal amounts of bone salt. Three animals were used for this experiment. All were placed on a scorbutic diet on the day of the operation and were given the following doses of vitamin C each day for the succeeding week: one animal received 8 mg. vitamin C by mouth, one animal received 2 mg. vitamin C by mouth, one animal received no vitamin C.

At the conclusion of the experiment one femur of each animal was macerated in 0·5 % potassium hydroxide for a week to remove the soft tissues. It was then washed, dehydrated, cleared and stored in oil of wintergreen (methyl salicylate). The second femur in each animal was fixed, decalcified, sectioned and stained.

An examination of the sections showed that some van Gieson staining trabeculae were present in the holes in the femora of all three animals, but there were obviously more in the animals receiving 8 and 2 mg. respectively of vitamin C. In the macerated bones of the 8 and 2 mg. animals there was no difference in intensity of staining or in the amount of bony material in the hole. They both had more bone in the hole than the animal which had been on the scorbutic diet without any supplement of vitamin C, and they showed a more intense staining of the bone around the hole than did the scorbutic animal. This latter observation was an indication that more bone salt was in fact being laid down as a result of the vitamin C given to the animals.

(3) A comparison of the hole in the macerated bone with the microscopical sections through the hole of the other femur of the same animal has therefore suggested that all trabeculae which formed were calcified. But the comparison *was* made by using two different femora albeit they came from the same animal. A method was required therefore which would permit one to see in microscopical sections whether a particular trabecula was calcified or not. It was found that pieces of the rib of a guinea-pig, bearing the costo-chondral junction could, after blocking in very hard celloidin, be sectioned without decalcification [Bloom, personal communication]. The sections could then be stained by von Kossa's silver nitrate method for demonstrating bone salt. By this means it is possible to see whether trabeculae are calcified or not.

As a preliminary test the costo-chondral junctions of a guinea-pig which had been on a scorbutic diet for 2 weeks and one which had received as much fresh green grass as it could eat during this period were treated as described above. As can be seen by inspecting Plate 1, figs. 1 and 2, there are many more bony trabeculae formed at the costo-chondral junction of the grass-fed animal than in that of the scorbutic animal. It may be noted, however, that there is an amorphous deposition of bone salt in the scorbutic animal in the region of the junction which is normally occupied by aligned cartilage cells. The cells are still there but they are not aligned and the bone salt appears to be deposited around their peripheries.

Three guinea-pigs were now placed on a scorbutic diet. Two were given supplements of 2 and 8 mg. respectively of vitamin C by mouth; the third animal received no supplement. At the end of 2 weeks costo-chondral junctions from all three animals were treated as above. On examination of the sections it could be seen that the zone of calcified trabeculae at the junction of bone and cartilage was widest in the animal receiving 8 mg. of vitamin C and smallest in the scorbutic animal: the calcified zone in the 2 mg. animal was intermediate between the scorbutic and the 8 mg. animals (see Plate 1, figs. 3–5). Some sections from these ribs were also treated with cobalt chloride and ammonium sulphide after the method of Gomori [1941] for demonstrating freshly deposited calcium phosphate. It was observed by this method that normal bone salt did not stain but that the freshly deposited bone salt at the junction of cartilage and bone did stain. The bone salt itself gradually darkened after the sections had been stained and mounted, but when they were first examined it could be seen that the area of freshly deposited bone salt was greatest in the animal receiving 8 mg. vitamin C and least in the scorbutic animal.

These results appear to suggest that vitamin C is associated with the process of calcification. Yet we should note that even in the scorbutic animals such trabeculae as are formed at the costo-chondral junctions appear to be as heavily calcified as those present in the animals receiving adequate vitamin C, although they are much fewer in number.
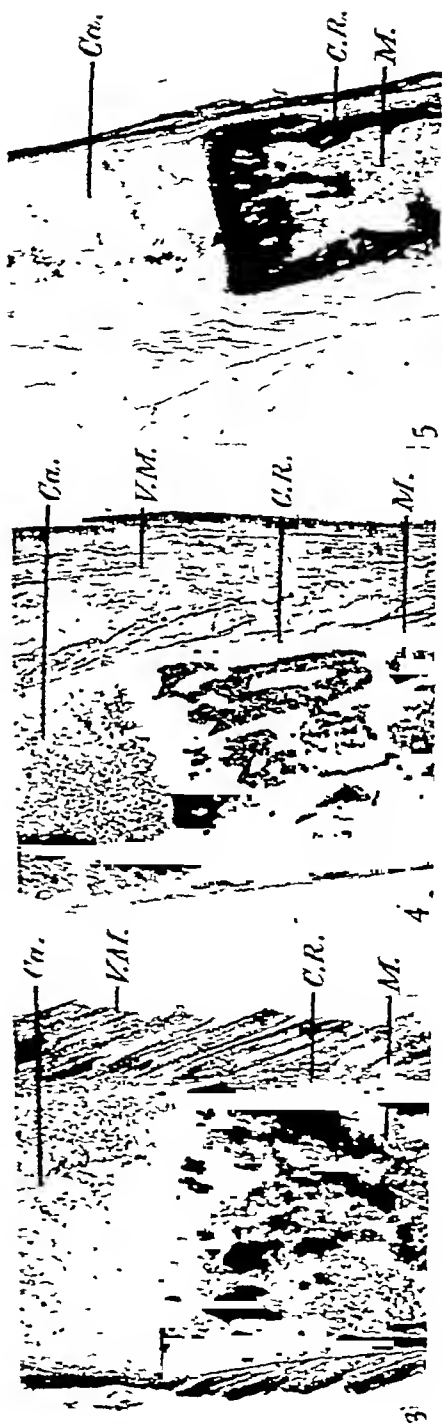
Figs. 1-5.

between the intensity of staining of the femora of the different animals. These results suggest that neither citrin (vitamin P) nor sodium citrate (substances of widely differing chemical nature) has any effect in the formation of the fibrous matrix of bone (no difference in the amount of osteoid formed) or in the deposition of calcium salts.

These conclusions have been subsequently confirmed by a similar experiment in which undecalcified sections of the costo-chondral junctions of six guinea-pigs were stained with von Kossa's silver nitrate method for demonstrating bone salt. Histological examination of holes bored in skulls of the same animals which had been healing for 1 week were also made. No difference in the calcification of the costo-chondral junctions or in the amount of bony trabeculae formed in the holes of the skull could be detected between those animals receiving only vitamin C and those receiving only citrin or sodium citrate.

It is of interest that Hartzell & Stone [1942] have found that vitamin P has no effect on wound healing.

(5) If vitamin C is associated with calcification, then one would expect the alkaline phosphatase activity of bones to decrease with scurvy. It has been shown by Bourne [1943] that in holes bored in the femora of scorbutic guinea-pigs little phosphatase can be demonstrated by Gomori's technique after 1 week's healing. But in holes bored in the femora of normal guinea-pigs, a concentration of phosphatase can be seen in the developing trabeculae by this time. One would not expect much phosphatase to be present in the tissue filling a hole in a bone until the trabeculae begin to develop and vitamin C deficiency, in any case, retards the formation of trabeculae, so the reduced amount of phosphatase in the scorbutic holes may simply be due to their absence. Nevertheless, in most animals the normal periosteum appears to react more strongly to Gomori's technique than does the periosteum of the scorbutic animal [Bourne, 1943]. But it appears that further information on the relationship between vitamin C deficiency and phosphatase activity is needed.

Four guinea-pigs were placed on a scorbutic diet for 3 weeks. Two were given daily supplements of 10 mg. vitamin C, each by mouth. At the conclusion of the experiment, pieces of rib, including the costo-chondral junction, were fixed in absolute alcohol subsequently embedded in celloidin and cut into sections 30μ thick without decalcification. Sections were treated by Gomori's phosphatase technique in which the sites of phosphatase activity were stained black. At the line of junction of bone and cartilage in the normal animals was a thick black band which was continuous with the periosteum. The endosteum of the trabeculae near the junction, the osteocytes and what appear to be the white cells of the bone marrow, all gave an intense black colour indicating that they had a high concentration of phosphatase. In the costo-chondral junctions of the scorbutic animals, the intensity of staining of the periosteum

seemed to be reduced only slightly, but the broad band of phosphatase at the junction was reduced to a narrow line.

Sections of the same bones were also incubated with Gomori's substrate mixture and sodium alizarin sulphonate (technique described by Bourne [1943]). By this method a red-coloured precipitate of calcium phosphate is laid down at the site of phosphatase activity. These preparations gave identical results with those obtained by the previous method.

These experiments suggest that vitamin C is associated in some way with phosphatase activity in the bones. It is of interest to note that while these experiments were in progress Gould & Schwachman [1942] published results obtained by a quantitative investigation which showed that bone phosphatase was reduced in scurvy. More recently, Schwachman & Gould [1942] have shown that there was a reduction of serum phosphatase in guinea-pigs on a scorbutic diet and that the phosphatase level returned to normal on the administration of vitamin C as ascorbic acid.

DISCUSSION

The results described in this paper have suggested that less bone salt is laid down by normal and regenerating bone in scorbutic animals than in those in which pure vitamin C is given. But the deposition of bone salt is an orderly and timed process in normal animals. That is to say, bone salt does not appear to be deposited (except in severe scurvy) until there is an adequate fibrous matrix to receive it. Urist & McLean [1941] have shown that as osteoid trabeculae are in the process of being formed they already have a deposit of bone salt. The production of the fibrous matrix of bone and the deposition of bone salt are therefore probably simultaneous processes. It would seem that as long as there is sufficient vitamin C to produce matrix then that matrix will be calcified. This is supported by the fact that in the costo-chondral junctions mentioned earlier, although the number of trabeculae is reduced in scurvy, those which were present still stained quite intensely with von Kossa's bone-salt method. The apparent failure of long bones to deposit bone salt in scurvy, therefore, may be due to the fact that no matrix has developed for its reception. It has been shown, however [Bourne, 1943], that bone matrix when formed appears to contain phosphatase, and since there is now evidence that vitamin C is associated in bone with the presence of phosphatase, it seems from this point of view that there may be some relation between vitamin C and calcification. The function of vitamin C in bone formation appears to be to facilitate the production, not just of bone matrix, but of bone matrix impregnated with phosphatase. There is no evidence at the moment that vitamin C can be regarded as a coenzyme of phosphatase in calcificatory processes. In fact Schwachmann & Gould [1942] find that there is no activation of serum phosphatase in vitro by vitamin C.

The apparent reduction of phosphatase activity in scurvy is therefore probably due to a reduction in the amount of bone matrix produced. Any matrix that is produced even in vitamin C deficiency will apparently have as much phosphatase as the matrix of a normal animal. Therefore it seems that if any matrix is formed at all in vitamin C deficiency it will be as heavily calcified as the matrix formed in normal animals. It is thus impossible to separate the functions of vitamin C as a substance facilitating bone-matrix formation and as a substance facilitating calcification, since these two processes are simultaneous.

SUMMARY

1. It has been shown that the deposition of bone salt in normal and regenerating bone is retarded in scurvy, but that pure synthetic vitamin C permits this process to take place.

2. That it is actually vitamin C and not some associated impurity that is responsible for this is suggested by the fact that the administration of vitamin P (citrin) and sodium citrate did not result in the formation of more osteoid trabeculae or the deposition of more bone salt than vitamin C alone.

3. The amount of phosphatase present in costo-chondral junctions was reduced in scorbutic animals.

4. It appears likely that one of the functions of vitamin C is to allow the production of a phosphatase-impregnated bone matrix upon which bone salt is immediately deposited.

5. Vitamin C may play some part in the formation or stabilization of alkaline phosphatase.

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EXPLANATION OF PLATES 1 AND 2

All figures ×20. Except where indicated all sections are taken through the centre of the costo-chondral junction and parallel with the long axis of the rib.

PLATE 1

Fig. 1. Undecalcified section of costo-chondral junction of rib of guinea-pig receiving ample diet of greenstuff. Stained von Kossa's method for demonstrating bone salt. Numerous calcified trabeculae may be seen extending from the junction.

Fig. 2. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet for 2 weeks. Stained von Kossa's method. Number of trabeculae greatly reduced, but the trabeculae which are present stain just as intensely as those in fig. 1.

Fig. 3. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet for 2 weeks'(see Exp. 4). Small number of trabeculae present.

Fig. 4. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet with a daily supplement of 2 mg. vitamin C. A greater number of trabeculae than in fig. 3 can be seen.

Fig. 5. Undecalcified section of costo-chondral junction of rib of guinea-pig receiving a scorbutic diet with a supplement of 8 mg. vitamin C. The calcified area at the junction may be seen to be wider than in either figs. 3 or 4. The apparent thinness of this section compared with the two preceding figures is due to the fact that it was cut parallel with the narrow axis of the rib, whereas the latter were cut parallel with the broad axis.

PLATE 2

Fig. 6. Undecalcified section of costo-chondral junction of guinea-pig on a scorbutic diet for 3 weeks but receiving a daily supplement of 10 mg. vitamin C. The section was treated by Gomori's alkaline phosphatase technique. A broad black band (A.) continuous on the right with the periosteum (P.) can be seen at the junction. These deep black areas indicate the site of the phosphatase activity. (The periosteum had been removed on the left of the section.) The marrow and the trabeculae also stain deep black, indicating the presence of appreciable amounts of alkaline phosphatase. The dark staining of the cartilage matrix is of no significance, since it is also present in the control (fig. 8).

Fig. 7. Undecalcified section of costo-chondral junction of guinea-pig receiving a scorbutic diet for 3 weeks. The line of phosphatase at the junction (A.) is reduced to a very thin band. The marrow and periosteum and trabeculae still stain intensely. The white area just above the junction is fibrous tissue. This is present because the rib had been fixed in a bent position while cut. Cartilage can be seen above it and below it at the junction.

Fig. 8. Undecalcified section of costo-chondral junction of guinea-pig's rib. Phosphatase control. Passed through Gomori's reagents for demonstrating freshly deposited phosphate but without prior incubation with phosphatase substrate. The cartilage stains black. There is a thin light-staining band of preformed, presumably freshly deposited, phosphate at the junction. The marrow stains very slightly. The endosteal lining of the bony trabeculae and the surfaces of the osteocytes in the trabeculae stain black. It should be noted that true bone has not stained.

Fig. 9. Undecalcified section of costo-chondral junction of guinea-pig receiving scorbutic diet for 3 weeks with daily supplement of 10 mg. vitamin C. Stained by modification of Gomori's alkaline phosphatase method, described by Bourne [1943]. In this method sodium alizarin

sulphonate is added to the substrate mixture and the calcium phosphate precipitated by the phosphatase activity is therefore red. A broad red band (*A*.) identical in position with the black band of fig. 6 may be seen. Nuclei of cartilage cells have stained and bone marrow is positive.

Fig. 10. Undecalcified section of costo-chondral junction of guinea-pig on a scorbutic diet for 3 weeks. The line of phosphatase is very reduced in size (*A*.) although it stains intensely. The marrow still stains. The staining of the osteocytes is very obvious in this and the preceding figure.

Explanation of lettering : *Ca*. cartilage, *T*. trabeculae, *C.R*. cortex of rib, *M*. marrow, *B*. deposit of bone salt in cartilage, *A*. line of phosphatase (see descriptions of figures), *V.M*. voluntary muscle, *P*. periosteum.

LIPOLYSIS AND FAT ABSORPTION

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According to the current hypothesis of fat absorption [Verzár & McDougall, 1936], lipolysis is an essential preliminary to absorption. The absorption of unhydrolysed triglyceride is regarded as an impossibility. If this were true, one would expect that neutral fat would give an identical picture to fatty acid during and after absorption. It can be shown that this is not the case [Frazer, 1938]. It is possible that fat is absorbed partly as a hydrolysed fraction and partly as unhydrolysed triglyceride and that lipolysis determines the route and destination of the absorbed fatty material. The object of this paper is to put forward further evidence in support of this conception of lipolysis in relation to fat absorption.

METHODS

Animals. Black and white adult rats from our own breeding stocks were used.

Materials. The fat used was olive oil B.P., the lipase preparation was 'Holadin' (Fairchild Bros.) and the sodium cetyl sulphate was obtained as 'Lissapol A' (I.C.I.).

Assessment of absorption. This was determined by analyses of the residual intestinal contents 6 hr. after administration of a known weight of fat. Absorption was confirmed by frozen sections showing the presence or absence of fat in the intestinal cells. The pathway taken by the fatty material after absorption was demonstrated by systemic and portal chylomicrographs. The eventual destination of the fat in the body was traced by the use of Sudan-stained fats which could be seen in the fat depots or the liver respectively. Human chylomicrographs were carried out under the standard conditions previously described [Frazer & Stewart, 1939].

* Sir Halley Stewart Research Fellow.

Results

The effect of added lipase in the rat

Fifty rats were used in these experiments. Groups of animals were fed with neutral fat to which was added an excess of active lipase. To control groups identical quantities of fat were fed with pancreatic extract in which the ferments had been destroyed by heat. The control groups showed the characteristic appearances found after neutral fat feeding. The animals which had extra lipase showed the changes usually associated with fatty acid feeding, namely, a fine granular deposit in the intestinal cells, no milkiness of the lacteals, a portal instead of a systemic lipaemia and deposition in the liver rather than the fat depots. The main findings are given in Table 1.

The effect of inhibition of lipolysis in the rat

Schulman [1941] showed that long-chain sulphates such as sodium cetyl sulphate inhibit the hydrolysis of ethyl butyrate by lipase. On testing the effect of this substance on the hydrolysis of triglyceride by lipase *in vitro*, it was found that 1/1000 sodium cetyl sulphate completely prevented lipolysis. This concentration must be slightly increased to ensure inhibition in the presence of bile.

Using fifty adult rats, groups of animals were fed upon olive oil mixed with a solution of 1/200 sodium cetyl sulphate while the control animals received a mixture of olive oil and water. The amount of fat absorbed was identical in the two groups, being slightly greater in those having cetyl sulphate. The histological picture, the chylomicrograph and the deposition of stained fat were all as found after triglyceride feeding. The results are tabulated below (Table 1).

TABLE 1. Results of ingestion by rats of neutral fat, fatty acid, neutral fat and lipase, and neutral fat and cetyl sulphate. Comparable amounts were absorbed in all groups. Average rate: 1 g. per 24 hr.

	Neutral fat	Fatty acid	Neutral fat + lipase	Neutral fat + cetyl sulphate
		Material ingested		
Intestinal cells	Large globules	Fine granules	Fine granules	Large globules
Lacteals	Milky	Nil	Almost clear	Milky
Systemic chylomicrograph	Normal max. 200	Low max. 20	Low max. 50	High max. 250
Fat depots	Heavy staining	Nil	Very little staining	Very heavy staining
Portal chylomicrograph	Low max. 20	Normal max. 200 +	Normal max. 200 +	Low max. 10
Liver	Small amount of staining	Heavy staining	Heavy staining	Very small amount of staining

In order to make certain that there was inhibition of lipolysis in the lumen of the rat's intestine under these conditions, samples of the intestinal contents were taken from some groups. This material was incubated and aliquots were

titrated at half-hourly intervals. There was no appreciable increase of fatty acid over a period of 6 hr. To further samples extra lipase was added but again lipolysis was found to be inhibited.

Effect of added lipase in man

Most of the observations made on rats cannot be checked in man. One striking change can, however, be demonstrated. The chylomicrograph may show differences between one individual and another, but any one subject
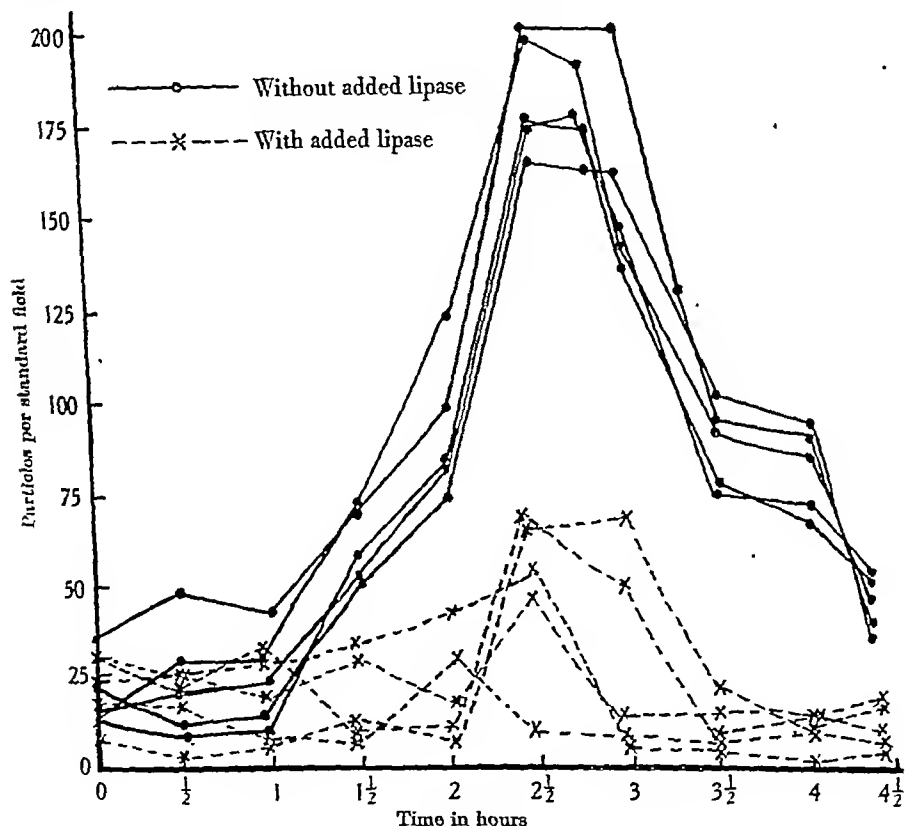
Fig. 1. Five normal human chylomicrographs following ingestion of 30 g. neutral fat with and without added lipase.

ingesting a standard amount of fat gives a reasonably constant lipaemic curve. If 30 g. neutral fat are ingested, the peak lipaemia will usually be in the region of 150–200 particles per field, but if to the 30 g. neutral fat is added some potent lipase (two 3 gr. capsules of 'Holadin'), the maximal systemic lipaemia is reduced by more than 75%. If the pancreatic extract is inactivated by heating before addition to the neutral fat it causes no such change

in the lipaemic curve. The depression of the post-absorptive lipaemia in a group of five human subjects by the simple addition of lipase is shown in Fig. 1. The average peak lipaemia in fifteen human experiments was 185 particles per field (range 100–300) after ingestion of 30 g. neutral fat, but when lipase was added to the 30 g. neutral fat the average peak lipaemia was only 45 particles per field (range 20–75).

DISCUSSION

If the breakdown of the triglyceride molecule is essential before it can be absorbed, the simple addition of lipase would not be expected to cause any profound change in the form, route or destination of absorbed fatty material. The first group of experiments, however, show that in the rat all these occur. The results fully confirm previous observations [Frazer, 1938] which were made after feeding with neutral fat or equivalent amounts of fatty acid respectively. While ingestion of neutral fat normally leads to a characteristic appearance of the intestinal cells, to milkiness of the lacteals, to a marked systemic lipaemia and to deposition of fat in the fat depots, the simple addition of potent lipase to the ingested neutral fat changes all these sequelae. Instead of large globules in the intestinal cells there are very fine granules, the lacteals remain almost clear, the systemic blood shows but a slight lipaemia, and deposition in the depots is much decreased. The portal blood and liver, which show only slight changes after neutral fat ingestion, exhibit marked lipaemia and deposition respectively if lipase is added to the neutral fat. The results following the ingestion of neutral fat and lipase are thus similar to those seen after the administration of fatty acid.

In the human subject it is only possible to check one of the essential observations. The normal chylomicrograph for any human subject under standard conditions is reasonably constant. It is possible to suppress almost completely the post-absorptive systemic lipaemia by the simple addition of lipase to the standard fat-containing meal. This observation correlates with the findings in the rat and it seems probable that the reason for the suppression of the systemic lipaemia is the diversion of the fat from the lymphatic pathway into the portal blood. It cannot be attributed to interference with, or delay in, the absorption of the fatty material.

The second group of experiments confirm the observations of Schulman that long-chain sulphates inhibit lipolysis. The inhibitory action of sodium cetyl sulphate in the rat's intestine has been carefully checked. There seems to be no reasonable doubt that fat is being absorbed in these experiments in the absence of any appreciable degree of lipolysis. It may be significant, however, that sodium cetyl sulphate is a strongly surface active substance and an excellent emulsifying agent. These experiments do seem to indicate that triglycerides may be absorbed without previous hydrolysis, although it

is probable that the presence of a surface active compound is important. From these experiments it appears that it is not essential to hydrolyse triglycerides for them to be absorbed.

These observations provide further support for the view that hydrolysis determines the mechanism of absorption, the route and destination of the absorbed fatty material. It is suggested that the current conception that hydrolysis of triglycerides is a necessary step in their absorption is incorrect, and that lipolysis should be regarded as a determining factor in the fate of absorbed fat and possibly as a means of providing essential raw materials for the synthesis of lecithin and the formation of soaps. This latter possibility will be discussed in a subsequent paper.

SUMMARY

1. Rats fed with neutral fat with added lipase show sequelae normally associated with the ingestion of fatty acid, such as fine granular deposition in the intestinal cells, portal rather than systemic lipaemia, and deposition in the liver instead of in the fat depots.

2. In human subjects under standard conditions, the systemic post-absorptive lipaemia can be almost entirely prevented by the simple addition of lipase to the fat-containing food.

3. The complete inhibition of lipolysis by sodium cetyl sulphate in rats does not prevent triglyceride absorption in amounts comparable with, or rather greater than, those absorbed by the control groups in the same time.

4. The significance of these findings is discussed and it is suggested that lipolysis is not an essential step in triglyceride absorption, but that firstly it determines the fate of absorbed fatty material and secondly it provides fatty acid for soap and phospholipid formation.

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THE EFFECT OF ANOXIA ON THE PRESSURE OF THE CEREBROSPINAL FLUID AND ON THE RATE OF ABSORPTION OF NORMAL SALINE SOLUTION FROM THE SUBARACHNOID SPACE OF DOGS UNDER ETHER ANAESTHESIA

By T. H. B. BEDFORD, *From the Department of Pharmacology, Manchester University*

The effect of anoxia on the pressure of the cerebrospinal fluid in cats anaesthe-tized with ether has been studied by White, Verlot, Selverstone & Beecher [1942], who found that prolonged uncomplicated anoxia was generally un-accompanied by any sustained increase in pressure. A marked increase in bra' volume was, however, observed in all their experiments. They consider that compensatory mechanism comes into play during prolonged anoxia by whic the volume of the cerebrospinal fluid is reduced. No observations appear t be available regarding the effect of anoxia on the rate of absorption of norm, saline solution from the subarachnoid space. It is proposed to describe series of experiments in which an attempt was made to study the effect (anoxia on the pressure of the cerebrospinal fluid and on the rate of absorptio of normal saline solution from the subarachnoid space of dogs under ethe anaesthesia.

EXPERIMENTAL PROCEDURE

The dogs were anaesthetized with ether administered through a tracheal tube A constant degree of pulmonary ventilation was maintained by means of pump. Anoxia was produced by administering a mixture of air and nitroge containing from 7 to 10 % oxygen. The mixture whose oxygen content wa determined by Haldane's [1920] method for gas analysis was stored in a larg Douglas bag and administered by connecting the bag to the inlet valve of th respiratory pump; the animal was prevented from breathing atmospheric ai by means of a simple valve system attached to the tracheal tube. Special care was taken to avoid any increase in the size of the dead space.

The pressure of the cerebrospinal fluid and the inflow of normal saline solution into the subarachnoid space were measured by methods which have

been described in detail in earlier communications [Bedford, 1935, 1936]. The rate of inflow was determined at a constant pressure of 300 mm. normal saline solution, except in two experiments where a study was made of the effect of variations in subarachnoid pressure on the rate of inflow during anoxia. The mean arterial pressure was recorded with a mercury manometer, from a cannula tied in the femoral artery. Animals in which the pressure did not exceed 100 mm. Hg were rejected and experiments were discontinued whenever the pressure fell below that level. The degree of oxygen saturation of the arterial blood was not determined in any of the experiments. The duration of an average experiment was 1 hr. 30 min.

RESULTS

The effect of prolonged anoxia on the pressure of the cerebrospinal fluid

The effect of anoxia on cerebrospinal-fluid pressure was studied in five animals. In two of the animals, the administration of a mixture of air and nitrogen containing from 7 to 10 % oxygen caused a temporary rise in the pressure of the cerebrospinal fluid. This increase in pressure was only moderate; it was accompanied by and appeared to be directly dependent on a rise in the systolic pressure in the femoral artery. The pressures regained their original levels after 9 min. in one animal and after 15 min. in the other. In the remaining three experiments anoxia caused no increase in the systolic arterial pressure, although the cerebrospinal-fluid pressure presented minor variations similar to those encountered in control animals anaesthetized with ether and receiving an adequate supply of oxygen. In all but one experiment the final pressure of the cerebrospinal fluid was somewhat lower than the pressure recorded during the initial control period. The effect of anoxia on the systolic arterial pressure and indirectly on the cerebrospinal-fluid pressure appeared to be related to the depth of the anaesthesia. The animals which displayed a rise in pressure were only lightly anaesthetized with ether while the remainder were more deeply anaesthetized.

The results of the five experiments are summarized in Table 1.

TABLE 1. The effect of anoxia on the pressure of the cerebrospinal fluid in five dogs anaesthetized with ether

Dog	Duration of anaesthesia in min.		Oxygen % in inspired mixture	Cerebrospinal-fluid pressure in mm. normal saline solution			
	Total	Anoxia		Control period	Onset	Maximum	Final
1	75	60	10	140	135	145	140
2	75	60	8·7	115	170	170	110
3	120	105	9·2	110	140	140	100
4	90	70	7·8	120	120	120	95
5	110	90	9·0	120	110	130	80

The effect of prolonged anoxia on the rate of absorption of normal saline solution from the subarachnoid space

The effect of prolonged anoxia on the absorption of normal saline solution from the subarachnoid space was studied in twelve animals. The duration of anoxia was never less than 1 hr., the maximum duration was 1 hr. 45 min. In ten experiments in which the average duration of anoxia was 1 hr. 8 min. no
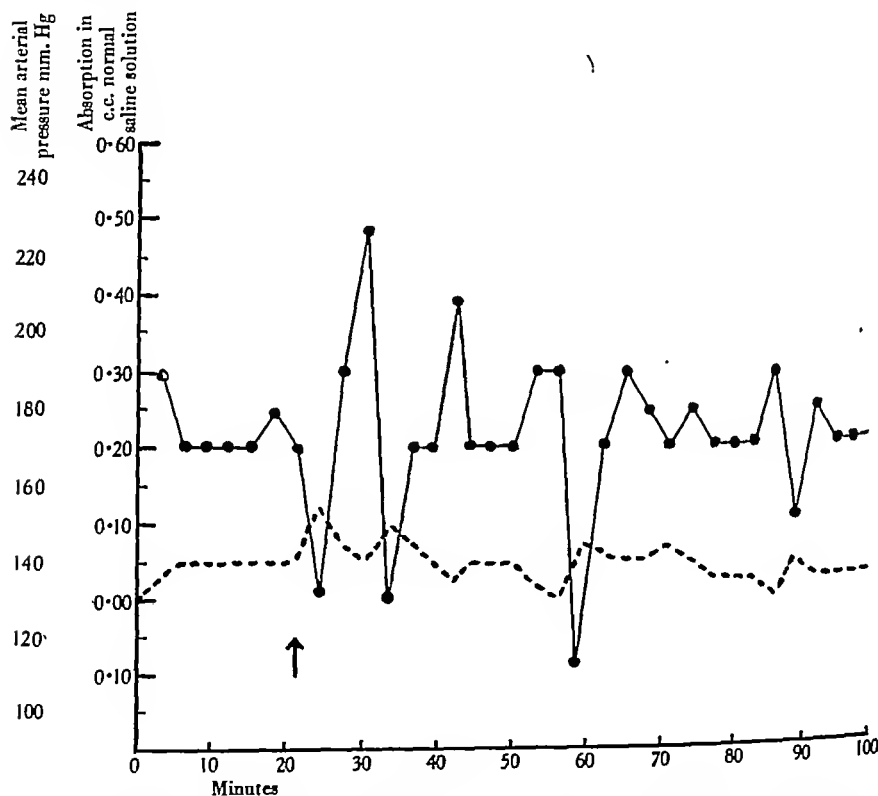
Fig. 1. The effect of inhalation of a mixture of air and nitrogen containing 8·7 % oxygen on absorption from the subarachnoid space. The animal began to inhale the mixture at the point indicated by the arrow. •——• inflow of normal saline solution; ——— mean arterial pressure. The arterial blood became markedly cyanotic in this experiment.

change was observed in the rate of absorption which could be attributed to the anoxia. Minor variations in inflow were, occasionally, observed, but they differed in no respect from those encountered in control experiments in which the animals were anaesthetized with ether and received an adequate supply of oxygen. In two of these experiments the onset of anoxia was accompanied by a sharp reduction in the rate of inflow which persisted for 9 and 12 min. respectively. The onset of this reduced rate of inflow was associated with an

equally abrupt rise in the systolic blood pressure in the femoral artery which persisted until the original rate of inflow was resumed when it fell to its original level. This period of reduced inflow was succeeded by one of increased inflow which persisted for 3 and 6 min. respectively; thereafter inflow proceeded at its original rate. A typical experiment is illustrated in Fig. 1.
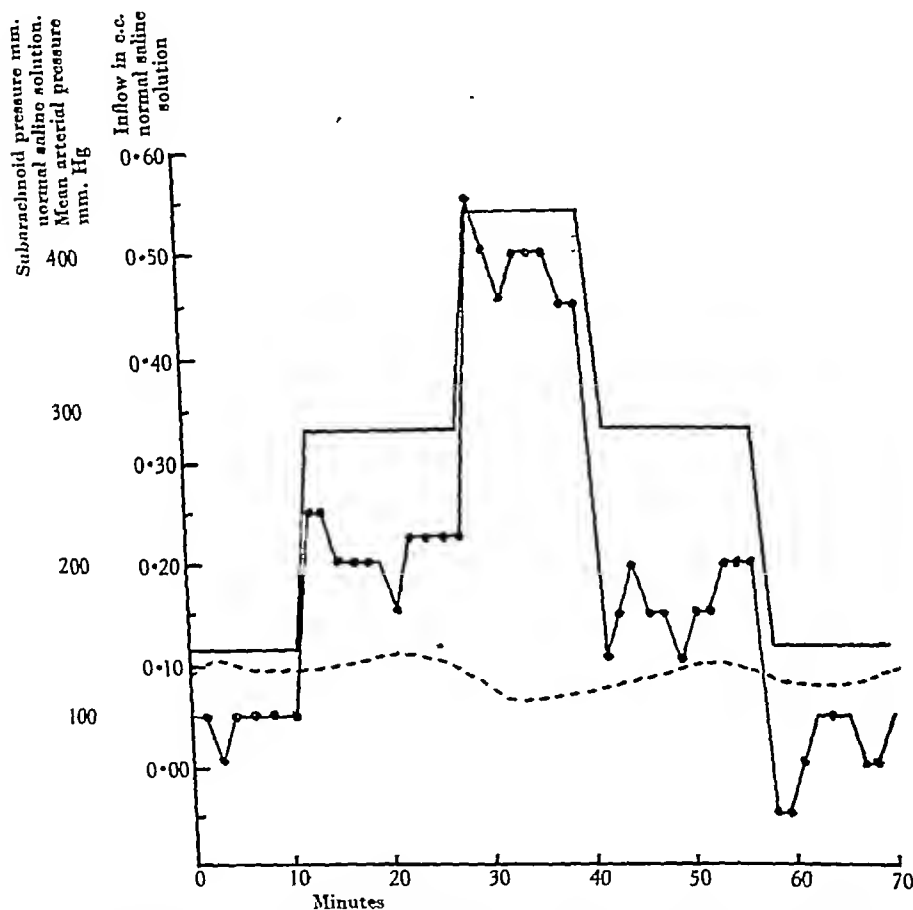
Fig. 2. The effect of artificial variations in subarachnoid pressure on the rate of absorption of normal saline solution in a dog inhaling a mixture of air and nitrogen containing 9·2 % oxygen. The subarachnoid pressure was set initially at a pressure level corresponding to that of the cerebrospinal fluid at the beginning of the experiment when the animal was breathing air. —— subarachnoid pressure; •—• absorption from subarachnoid space; —— mean arterial pressure.

In the remaining two experiments the state of anoxia was associated with a reduction in the rate of inflow. In one experiment a gradual reduction appeared after 30 min. The decline progressed and at the end of the experiment, 48 min. later, inflow was proceeding at approximately half the rate which

obtained during the initial control period. The blood pressure remained steady throughout this experiment. In the other experiment an abrupt falling off in the rate of inflow was observed after 1 hr. 15 min. Inflow then proceeded at half its original rate until the end of the experiment 15 min. later. The systolic pressure on the femoral artery showed only minor variations which bore no relation to the onset of reduced inflow. A careful examination after death of the brains of these two animals failed to reveal any abnormality.

A study was made in two animals of the effect of variations in subarachnoid pressure on the rate of inflow of normal saline solution during anoxia. The rate of inflow was recorded for a period of not less than 12 min. at each new pressure level so as to obtain a reasonably accurate average figure. The findings did not differ in any respect from those obtained in experiments on animals receiving an adequate supply of oxygen [Mortensen & Weed, 1934; Bedford, 1938]. The results of one of these experiments are indicated in Fig. 2.

A marked degree of cyanosis of the arterial blood was constantly observed during anoxia produced by inhalation of mixtures of nitrogen and air containing from 7 to 10 % oxygen.

DISCUSSION

It is evident from the above experiments that a rise in the pressure of the cerebrospinal fluid does not accompany the anoxia produced by inhalation of mixtures of air and nitrogen containing from 7 to 10 % oxygen. Evidence is also presented that the rate of inflow of normal saline solution, at a constant pressure, into the subarachnoid space is generally uninfluenced by this degree of anoxia; a diminution of inflow was observed in only two experiments out of a total of twelve. Anoxia failed to produce any change in the effect of artificial variations in subarachnoid pressure on the rate of inflow of normal saline solution into the subarachnoid space. The findings of White et al. [1942] that anoxia produced by the inhalation of 8–10 % oxygen fails to produce any sustained increase in the pressure of the cerebrospinal fluid are, in the main, confirmed, although these workers occasionally observed a marked rise in the pressure of the cerebrospinal fluid which could not be correlated with any change in the systemic arterial blood pressure. They found, however, that anoxia invariably caused a marked degree of swelling of the brain and they were compelled to postulate a reduction in the total volume of the cerebrospinal fluid to account for the frequent occurrence of a low cerebrospinal-fluid pressure at the end of their experiments. They consider that a reduction in the volume of the cerebrospinal fluid may be brought about in several ways: (1) an increase in the rate of absorption by the normal channels secondary to an elevated hydrostatic pressure; (2) direct imbibition of fluid by the anoxic brain tissue; (3) decreased production of cerebrospinal fluid. A swollen brain, which White et al. observed in all their experiments, would ultimately obstruct

the subarachnoid channels. Initially this could be compensated by a rise in the pressure of the cerebrospinal fluid, but the rise would need to be a sustained one if the production of cerebrospinal fluid remained unchanged. For a similar reason it is improbable that imbibition of fluid by the brain could function as a compensatory mechanism. It is difficult, therefore, to account for a normal cerebrospinal-fluid pressure in association with a swollen brain on any basis other than a reduction in the formation of cerebrospinal fluid.

In the experiments now under consideration the rate of inflow of normal saline solution at a constant pressure of 300 mm. normal saline solution was uninfluenced except perhaps in two experiments by prolonged anoxia. It should, however, be borne in mind that the rate of inflow of normal saline solution into the subarachnoid space is probably not a true index of total absorption; the relative rates of production and absorption need consideration. Nevertheless, Mortensen & Weed [1934] have shown that production and absorption at the normal pressure level of the cerebrospinal fluid balance each other with minor fluctuations from time to time on either side. It is probable, therefore, that the inflow observed when the subarachnoid pressure is raised above this level represents absorption but not the total absorption of fluid from the subarachnoid space. It would appear that, with the possible exception of two experiments, anoxia was without influence on absorption. Additional evidence for this conclusion is provided by the observation that artificial variations in subarachnoid pressure were accompanied by changes in the rate of inflow indistinguishable from those observed in experiments on animals receiving an adequate supply of oxygen.

It is concluded that any swelling of the brain that may be induced by anoxia is moderate in degree and usually insufficient to obstruct the circulation of the cerebrospinal fluid. It is, therefore, unnecessary to postulate a reduction in the rate of formation of cerebrospinal fluid to account for the normal pressure of the cerebrospinal fluid encountered after prolonged anoxia.

SUMMARY

1. A study has been made of the effect of prolonged uncomplicated anoxia on the pressure of the cerebrospinal fluid, the absorption of normal saline solution from the subarachnoid space, and the response of absorption to artificially induced variations in subarachnoid pressure.

2. Anoxia was without influence on the pressure of the cerebrospinal fluid. Absorption also was uninfluenced except in two experiments out of a total of twelve where a reduction was observed. The effect of variations in subarachnoid pressure on absorption was studied in two experiments and was found to be accompanied, in each instance, by changes in the rate of absorption indistinguishable from those observed in experiments on animals receiving an adequate supply of oxygen.

3. It is concluded that any swelling of the brain that may be induced by anoxia is probably moderate in degree and usually insufficient to obstruct the circulation of the cerebrospinal fluid.

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THE OUTPUT OF CORTICAL HORMONE BY THE MAMMALIAN SUPRARENAL

By MARTHE VOGT, *From the Pharmacological Laboratory, the College of the Pharmaceutical Society, University of London*

Substitution therapy in suprarenal insufficiency is based on the beneficial effects observed, in man or in animals, with extracts of cortical tissue or with synthetic compounds, and not on any knowledge of the physiological activity of the suprarenal cortex. No data are available on the amount of cortical hormone present in the blood, and information is lacking on the rate of secretion by the suprarenal cortex and its possible variations under different conditions. It is the object of the present paper to supply such information. It was obtained by collecting the blood from the suprarenal veins of different mammals and assaying its cortical activity. The experiments are restricted to anaesthetized animals subjected to a severe abdominal operation.

METHODS

(1) *Assay of cortical hormone*

The method used for the assay of cortical hormone is that devised by Selye & Schenker [1938]. For the present purpose it has three essential merits: first, it is more sensitive than most other methods; secondly, it does not require weeks of observation of the test animals and, consequently, deterioration of the solutions during the period of assay need not be feared; thirdly, it is not specific for any particular fraction of the cortical steroids and will, therefore, give a better survey of suprarenal activity than a method which is particularly sensitive to, for example, steroids affecting mostly cell permeability or compounds acting mainly on carbohydrate metabolism. Employing this method, Kendall [1941] found comparable potencies for corticosterone, compound E and the 'amorphous fraction', and Zarrow [1942] demonstrated the efficacy of desoxycorticosterone acetate, provided it was administered some hours before the exposure, thus allowing time for absorption.

The principle of the method had been suggested by Hartman, Brownell & Crosby [1931]. Young suprarenalectomized rats are submitted to stress from

cold environment. Selye & Schenker expose the animals to temperatures of +2–4° C. on the day after the operation. The rats are divided into groups and given different amounts of cortical extract hypodermically during the first hours of exposure. The survival time is observed and seen to increase with the quantity of extract administered.

Details of the method, as I have used it, are as follows: The groups were made up of nine to ten rats of as nearly as possible the same age and weight. As the survival time of the rats increases with weight and decreases, within certain limits, with the age at which a given weight is attained, the groups had to be matched carefully with regard to these two factors. Litter mates were evenly distributed among the groups, and the mean weight of each group was always made equal. Although an effect of sex on sensitivity is not certain, the same ratio of males to females was used throughout the groups.

It is not possible to use rats of different colonies in one assay. Vigorous strains are more susceptible to cortical hormone than weak ones; the latter require very large quantities of extract to protect them. For one colony of Wistar rats, ages from 23 to 30 days and weights between 38 and 60 g. proved workable, whereas for another strain (Wistar rats, Dr K. H. Coward's colony, reared under strictly constant dietary conditions) suitable figures were 19–23 days and 38–45 g. The mean survival time for rats of this last colony was about double that of the first.

Suprarenalectomy was carried out by the lumbar route from a midline incision through the skin. Pentobarbitone combined with ether were used as anaesthetics. The rats were given a few drops of 25 % glucose solution soon after the operation. They took food overnight, usually gained in weight, and were lively and fit the next morning when the experiment was about to start.

For the test, the rats were distributed into individual numbered cages early in the morning, given their first injection and simultaneously placed in a large refrigerator. At intervals of an hour and a half further injections were made, their total number being either four or five. The doses were injected as rapidly as possible, all the cages being removed from and replaced into the refrigerator simultaneously. From the 5th or 6th hour after the beginning of exposure, a rapid inspection of the rats was carried out every half-hour, any deaths recorded, and these observations continued till all or nearly all rats had died.

Calculation of the mean survival time was made for each group. Sometimes, however, when observations had not been continued till all rats in a group had died, the percentage of deaths observed at the half-hourly intervals was plotted against time on probability paper and the mean survival time found graphically.

Since the rats in each group were not distributed at random, but carefully selected, so as to have in every group representatives of the same litter, ages, weights, and sexes, the test for 'significance' of the result was based on the

standard deviation of the *differences* in survival times between corresponding pairs of rats in any two groups and not on the standard deviation of the survival times themselves. By dividing the standard deviation of the differences by the root of the number of pairs, the standard error ϵ of the mean difference D was obtained and the ratio D/ϵ gave the usual measure for 'significance', the probability that a result was due to chance being determined by means of Fisher's table [1941].

Fig. 1 is a dose-response curve obtained by injecting three groups of ten rats with increasing doses of a cortical extract ('eucortone', Allen and Hanburys). Each rat had five injections containing 0·012 ml. eucortone in group 1, 0·03 ml. in group 2, and 0·075 ml. in group 3. In the graph, the logarithms of
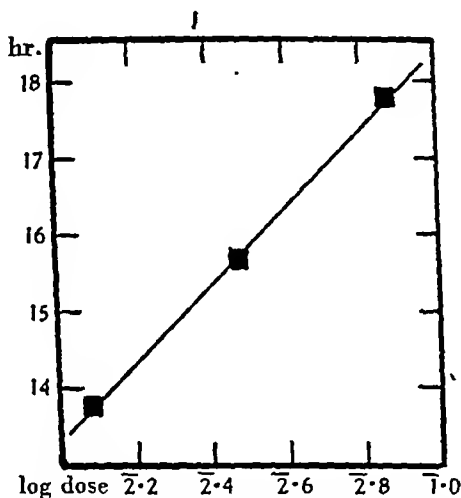
Fig. 1. Dose-response curve. Ordinate: mean survival time (hours) of groups of ten rats.
Abscissa: log dose (ml. cortical extract per rat and injection).

these doses are plotted as abscissae against the mean survival times (13·75, 15·67 and 17·81 hr.) of the three groups as ordinates. As shown in the figure, the three points lie in a straight line, and indicate that the response is proportional to the logarithm of the dose. This result is similar to that obtained by Bülbring [1937] on the survival times of suprarenalectomized drakes. A calculation of the standard errors of the differences in survival times as outlined above led to the following result: the probability that the mean difference in survival time between groups is due to chance is 1:20 for groups 1 and 2, and 1:50 for groups 2 and 3. In other words, the differences obtained with the three doses, though significant, are not sufficiently large to warrant further reduction of the differences between the doses. The doses had been so selected as to increase by the factor 2·5, and the result of the calculation of the error shows that smaller differences between doses would not have been detected

with certainty. This result was confirmed in several other experiments by the observation that the increase in survival time obtained by doubling the basal dose was not significant, whereas that resulting from trebling it was. In this respect the method compares unfavourably with assays on drakes [Bülbring, 1937], in which doses differing by 50 % are distinguishable. Its advantage is its high sensitivity; the total dose required per rat is about 1/25th of that necessary per drake. In Fig. 1, for instance, the smallest dose used for one rat is 5×0.012 or 0.06 ml. extract; the survival time with this dose was 5·75 hr. longer than with saline and the response, therefore, considerably above threshold.

If two groups of rats of the same age and average weight are taken from the same colony and treated with the same dose of cortical extract or injected with the same volume of saline in two consecutive experiments with, as far as one can tell, identical environmental conditions, the mean survival time will nevertheless be different. Consequently, in each assay of cortical activity the survival time without treatment must be established for a group of rats injected with saline only, and the susceptibility to hormone by at least one group given a cortical extract used as 'standard'. If quantitative assay of differences between several unknown samples is desired, two groups of rats given two different doses of 'standard' are required.

(2) Collection of suprarenal blood

Collection of the blood leaving the suprarenals was carried out by Verney's modification of the method devised by Feldberg & Minz [1934] in their work on the acetylcholine content of the suprarenal effluent. On a heparinized dog, blood is drained from the left suprarenal gland. For this purpose, the branches of the left lumbar vein are tied, a loose ligature is looped round its entry into the vena cava, and a cannula introduced into the vessel about 1 cm. lateral to the suprarenal gland. The cannula is connected by a short rubber tubing to one arm of a T-piece pushed through a small stab wound in the animal's left flank. The stem of the T-piece is connected by tubing to a cannula introduced into the left jugular vein. The loose ligature around the caval junction of the vein is tied, and blood may now either be drained from the second arm of the T-piece into a flask, if the tubing leading to the jugular vein is clamped, or be allowed to flow back into the animal, if the second arm of the T-piece is occluded.

Modifications of this technique had to be applied if mammals other than dogs were used. For the cat, the colon was removed, aorta and cava were tied below the renal vessels, the renal pedicles were ligated and a cannula was introduced into the cava, caudad to the adreno-lumbar veins, which were tied at the lateral edge of the gland. During the collection of suprarenal blood, the cava was occluded above the suprarenals. The same operation was carried out in

the rabbit, but it was preceded by evisceration. The volume of the blood samples required for a test was about 30 ml. In order to obtain this quantity from an eviscerated rabbit, blood from another rabbit had to be infused intravenously while the suprarenal sample was being collected.

In the pig and the goat the left renal vein receives most of the blood from the left suprarenal; accordingly, the left renal artery was tied, the renal vein cannulated, its caval end ligated and the cannula connected to a T-piece, which allowed the blood to return to the jugular vein in the goat and to the subclavian vein in the pig.

Ether followed by chloralose intravenously were the anaesthetics used for all the animals.

The blood draining from the suprarenals was collected in centrifuge tubes cooled in ice water. The red cells were separated off and the plasma was assayed by subcutaneous injection into groups of rats as described above. In most experiments the plasma was assayed 14 hr. after collection. Later, however, tests were frequently carried out 40 hr. after obtaining the blood, as the results seemed unaffected by that delay. As soon as the red cells had been centrifuged off, all plasma samples were placed into a refrigerator where they remained until use.

(3) *Effect of adrenaline on the assay of cortical hormone*

Some of the experiments were carried out on animals in which the splanchnics were intact, and the blood samples were thus liable to contain adrenaline due to reflex stimulation of the splanchnics by the operative procedures. It was, therefore, necessary to know whether the presence of adrenaline in the plasma interfered with the assay of cortical hormone. Groups of rats were treated with suitable amounts of eucortone and the survival times compared with those of other groups given the same quantity of eucortone to which adrenaline had been added up to a concentration of 1:50,000. Similar comparisons were made between rats injected with saline and rats given adrenaline solutions 1:50,000. Adrenaline did not significantly influence the survival times observed in the rats. These results are in agreement with earlier observations by Hartman *et al* [1931]. They render any correction for the adrenaline content of the plasma unnecessary.

RESULTS

(1) *Presence and estimation of cortical hormone in suprarenal venous blood*

Blood from the suprarenal veins was assayed for cortical hormone in a large number of dogs, several cats, two goats, one rabbit and one pig. A considerable cortical activity was invariably found, whereas power to prolong the life of suprarenalectomized rats was lacking in all control plasmas obtained from the main arteries of the body or the right side of the heart. The only exception was a slight protection afforded to the rats by arterial blood of the cat (see p. 354

The assay was uncomplicated in the dog and the cat as their plasma had hardly any toxic effect on suprarenalectomized rats. Injections of plasma from the other animals, however, proved highly toxic for the rats, and the method of estimation had to be modified accordingly. An example of two typical experiments, one on a dog and one on a goat, will be given first.
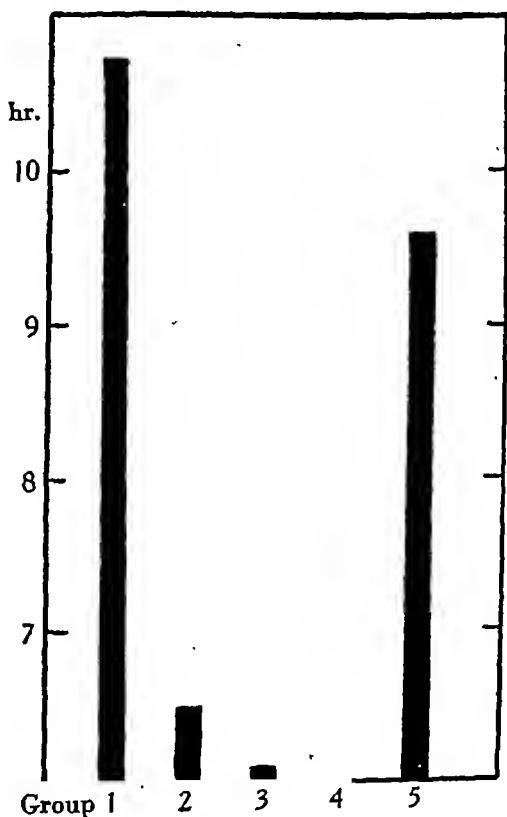
Fig. 2. (Exp. 13, dog.) Ordinate: mean survival time (hours). Number of group at the bottom of each column. The rats received per injection: group 1, 0·2 ml. suprarenal plasma; group 2, 0·5 ml. NaCl; group 3, 0·5 ml. plasma from heart and femoral artery; group 4, 0·5 ml. 10 % eucortone in NaCl; group 5, 0·5 ml. 10 % eucortone in heart plasma.

Example of an assay in the dog. In the dog (Exp. 13, Table 1) chosen as an example, the sample of suprarenal blood was collected while both splanchnic nerves were intact, the blood pressure being over 100 mm. Hg and the flow through the left suprarenal about 0·2 ml. (0·1 ml. plasma) per min. (This is unusually small and is due to the small size of the dog—5 kg.) Of the plasma so obtained, four injections of 0·2 ml. were given to a first group of rats; their mean survival time was 10·7 hr. This time is represented in column 1 (Fig. 2); the other columns are the mean survival times obtained with other solutions

on the same batch of rats. Thus, a control group treated with 0·9 % NaCl solution survived for 6·5 hr. (column 2). The difference of the two means is statistically significant. Column 3 represents the effect of blood obtained from other parts of the vascular system. The rats of that group were given four injections of 0·5 ml. of plasma prepared from blood collected from the right side of the heart and the femoral artery. The survival time hardly differs from that of the saline-treated group. No detectable amount of hormone, therefore, is present in heart blood and arterial blood of this dog, although the quantity of plasma tested was $2\frac{1}{2}$ times as large as that of the suprarenal sample.

In group 4, the potency of a commercial suprarenal extract ('eucortone', Allen and Hanburys) was tested by four injections of 0·05 ml., diluted 1:10 with saline. If we compare the protection thus obtained (column 4) with that afforded by 0·2 ml. suprarenal plasma, we find only an insignificant difference. We are not justified, however, in concluding from this comparison that 0·2 ml. plasma contain as much active principle as 0·05 ml. eucortone, without first ascertaining that non-specific constituents of the blood are not interfering with the action of the eucortone. This was done in the following way: a fifth group of rats was given the same amount of encortone as group 4, dilution, however (1:10), being made with dog's heart plasma instead of saline. Column 5 shows the survival time to be equal (within the errors of the method) to that of group 4. Direct comparison is thus permissible of the activity of the suprarenal plasma with that of encortone solution in saline.

From the results of Fig. 2 we are able to express the output of cortical hormone into the blood in terms of the extract 'eucortone'. In order to make the lowest possible estimate of the glandular activity, we shall assume that all the hormone is present in the plasma and none has entered, or been adsorbed to, the red cells. One ml. eucortone is the extract of 75 g. gland, but its potency varies according to the batch. The sample used in this experiment had only two-thirds of the activity of the most potent batches encountered. For our calculations, 1 ml. eucortone will, therefore, be assumed to represent the activity of 50 g. gland only. It follows that the potency of 1 ml. plasma corresponds to that of 12·5 g. gland. In this 5 kg. dog, the output per minute from one suprarenal (0·1 ml. plasma) was equivalent to the activity contained in 1·25 g. gland, and, per kg. body weight, to that contained in 0·25 g. gland.

These values are obtained on the assumption that the potencies represented by columns 1 and 4 or 5 are identical. In fact, column 1 probably represents a somewhat higher value than column 4, and the figures for the yield are likely to be underestimates.

It was confirmed in many experiments that dog's plasma, taken from any part of the body, has negligible toxic effects on suprarenalectomized rats. We need, therefore, not fear the interference of substances present in the blood when estimating the content of cortical activity. Cat's plasma was similar to dog's

plasma in that respect, but it differed, in some instances, in affording a slight protection to the rats. The effect was statistically significant but very slight. An example will be given later. Entirely different effects, however, were obtained with plasma from the cow, the goat, the rabbit and the pig. This is illustrated by Fig. 3 (Exp. 23, Table 1, carried out on a young goat in a similar way as the experiment on the dog represented in Fig. 2).
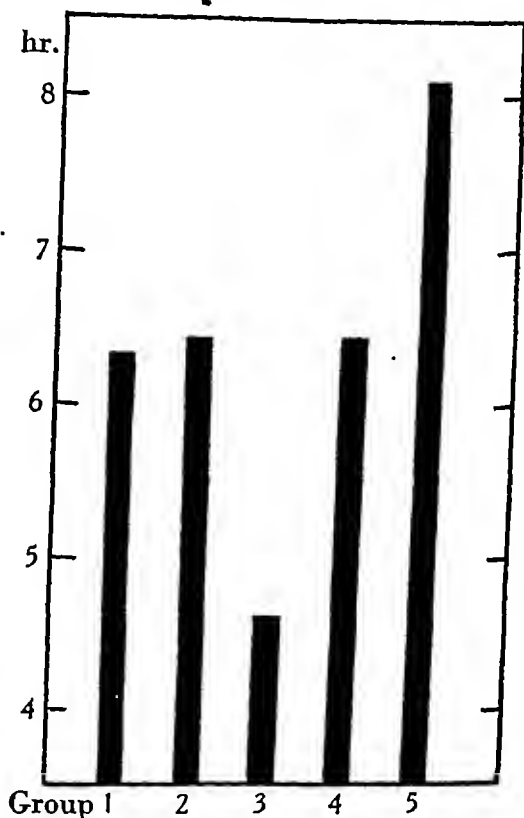
Fig. 3. (Exp. 23, goat.) Ordinate: mean survival time (hours). Number of group at the bottom of each column. The rats received per injection: group 1, 0·4 ml. suprarenal plasma; group 2, 0·4 ml. NaCl; group 3, 0·4 ml. plasma from heart and femoral artery; group 4, 0·4 ml. 3·75 % eucortone in heart plasma; group 5, 0·4 ml. suprarenal plasma after splanchnotomy.

In Fig. 3, column 1 represents the survival time obtained with a sample of suprarenal plasma, the splanchnics being intact, the blood pressure 68 mm. Hg and the suprarenal flow 4·2 ml. per min. (2·1 ml. plasma). The next column gives the result of injecting saline instead of plasma. The survival time is the same. The third column illustrates the effect when a mixture of arterial and heart plasma is given in the same quantity (0·4 ml. per injection) as suprarenal plasma: the survival time is much shorter than with saline. The difference

between the two results is statistically significant. The fourth column represents the survival time resulting from treatment with 3·75 % eucortone added to the plasma used for group 3; the survival time is prolonged beyond that of group 3 and reaches the figure observed in groups 1 and 2.

The experiment demonstrates a strong toxicity of goat's plasma for suprarenalectomized rats kept in the cold. In another similar experiment, even quantities as small as 0·2 ml. per injection reduced the survival time of rats considerably beneath that of saline injected controls. This toxicity is overcome by adding cortical extract to the plasma. In the example given in the figure, the quantity of eucortone added was just sufficient to raise the survival time to the level obtained with a first sample of suprarenal plasma, and we are thus enabled to state that this plasma has the same potency as a 3·75 % solution of eucortone.

From a calculation similar to that applied earlier on in the dog, it follows that the left suprarenal of the goat which gave the results shown in Fig. 3 produced, before splanchnotomy, a quantity of hormone corresponding to 6 g. gland per min., which is 0·4 g. gland per min. for each kg. of body weight. As the suprarenal weighed 0·55 g., the gland produced per minute 11 times the amount which can be obtained by its extraction.

The activity of a second sample of plasma, collected in the same experiment after section of the left splanchnic, is represented in column 5. The survival time is much longer than that in any other group, including the saline-treated control; this shows that a sufficiently high degree of cortical activity will not only neutralize the toxicity of goat's blood but render the plasma highly beneficial for suprarenalectomized rats.

As far as can be told from the small number of experiments carried out with plasmas from cows, pigs, rabbits and goats, their toxicity for suprarenalectomized rats is of the same order. We have just seen that the addition of 3·75 % eucortone to a sample of goat's heart plasma neutralized its toxic properties so that the mixture acted like saline. In another experiment, the same amount of cow's plasma was made innocuous by addition of 3 % eucortone. Rats treated with this solution survived for 11 hr. and so did a control group injected with saline; a third group, however, which was given 3 % eucortone diluted with saline instead of with plasma lived for 13·7 hr. (The difference between 11 and 13·7 hr. is statistically significant.) In a first test with pig's plasma, the addition of 8 % eucortone made the plasma just harmless, and in another test 10 % were far in excess for the purpose of 'neutralizing' its toxic properties; a similar result was obtained in the rabbit.

It follows from these observations that, in assaying the cortical activity of plasma with rabbits, pigs, goats and cows, it is not sufficient to compare the potency of the sample with that of solutions of a cortical preparation in saline. Comparison has to be made with solutions of the known extract in plasma

obtained from any part of the body other than the suprarenals. Rats injected with that same plasma without the addition of cortical extract take the place of the saline-treated controls in other experiments.

TABLE 1. Output of cortical hormone by the suprarenal gland of one side, per min. per kg. body weight. The output is expressed by the amount of suprarenal tissue ('g. gland') which yields, on extraction, the same activity

No. of exp.	Animal used	Conditions	'g. gland' per min. per kg.	Output per min. in terms of 'content' of own gland
10	Dog	Splanchnics intact	0·26	—
13	,,	,,	0·3	—
15	,,	,,	0·53	—
29	,,	Splanchnics intact, eviscerated	0·75	17 times
14	,,	Left splanchnic cut	>0·46	—
17	,,	,,	0·38	—
18	,,	,,	c. 0·5	c. 9 times
20	,,	,,	0·38	4 ,,
34	,,	Left splanchnic cut, eviscerated	>0·41	>4·5 ,,
37	,,	,, ,,	0·95	13 ,,
39	,,	,, ,,	2·0	29 ,,
42	,,	,, ,,	0·62	10 ,,
46	,,	,, ,,	c. 0·5	c. 11 ,,
57	,,	Splanchnics cut, eviscerated	0·39	5·7 ,,
51	Cat*	,, ,,	⋚1·0*	⋚7·5 ,,
55	,,	Splanchnics intact, eviscerated	0·75*	5 ,,
26	Pig	Splanchnics intact	⋚0·41	⋚5 ,,
23	Goat	,,	0·40	11 ,,
33	,,	Left splanchnic cut, eviscerated	>0·48	>16 ,,
65	Rabbit*	Splanchnics intact, eviscerated	2·0*	13 ,,

* Unlike the other figures, those for the cat and the rabbit represent the output not of one, but of both suprarenals.

The results obtained in these two experiments are no exceptions, as may be seen from Table 1, which summarizes all the experiments in which the activity of suprarenal plasma was compared with that of 'eucortone'. The amount of hormone released from one suprarenal gland, per min. per kg. body weight, varies in different experiments but is of the same order in the five mammals tested. The yield ranges between 0·26 and 2·0 'g. gland'. In the last column, the suprarenal output is compared with the hormone 'content' of the animal's own suprarenal, 'content', however, signifying the activity which can be extracted from the gland as distinct from its real content, which is unknown. In all instances in which it was possible to make this calculation, the minute output was many times higher than the 'content' of the secreting gland. In half of the experiments, the amount given off per minute was at least 10 times as high as the 'content' of the animal's gland, and in one instance it was nearly 30 times as high.

This considerable rate of hormone production also becomes apparent, if comparison is made between the concentration detected in plasma prepared from suprarenal blood, and the quantity obtainable by extraction of 1 g. gland tissue. This calculation has been carried out for experiments on dogs (Table 2).

TABLE 2

No. of exp.	1 ml. suprarenal plasma equivalent to 'g. gland'	No. of exp.	1 ml. suprarenal plasma equivalent to 'g. gland'
10	2·1	37	10·0
13	12·5	39	10·0
15	5·0	42	6·5
17	3·8	46	8·3
20	2·2	52	6·2
29	10·0	57	4·8

It is obvious that 1 ml. of plasma always contained much more hormone than can be obtained by extraction of 1 g. of glandular tissue. In one-third of the experiments the ratio was 10:1 or greater.

(2) *The effect of blood flow and blood pressure on the output of cortical hormone*

In the interpretation of results bearing on the question whether certain conditions modify the output of cortical hormone, the limited accuracy of the method has to be taken into account. Changes can, as a rule, only be ascertained if they are large, e.g. threefold increases or reduction to one-third of the basal value. The effect of a particular factor can be tested by comparing the yield in several experiments differing from each other with regard to that factor. Such information, based on the foregoing experiments, will be described here. Experiments in which the output is measured in consecutive samples obtained from the same animal under varying conditions will be reported in another paper.

(i) *Blood flow.* The weight of the suprarenals varied between 0·4 and 1·3 g., their blood flow, however, between 0·2 and 8·2 c.c./min. The figures apply to one gland only, except in the case of the cat and the rabbit. In spite of this wide range of blood flows, no correlation was found between yield of hormone and flow through the gland. In fact, when the blood flow changed considerably during the same experiment, the concentration of cortical activity varied inversely with the flow, the hormone production per minute remaining comparatively unchanged. An example was given in Fig. 3. Between the collection of the first (column 1) and the second (column 5) sample of suprarenal plasma, the blood flow through the gland had fallen to one-third of its original value. The fall was partly due to the fact that the left splanchnics had been severed after the collection of the first sample. The considerable increase in hormone concentration is apparent in the figure. It may well be an expression of an unchanged rate of hormone production and, therefore, of the independence of hormone output on blood flow. This argument is, of course, only valid if section of the splanchnic in itself does not alter the cortical activity. Evidence for that assumption will be published later.

(ii) *Arterial blood pressure.* During the collection of suprarenal blood samples in different experiments, the mean arterial pressure varied from 25 to 132 mm. Hg. There was no correlation between blood pressure and amount of hormone

secreted per unit of time. In order to obtain a high concentration of active principle, it is, therefore, best to work at a low arterial pressure and a correspondingly small blood flow.

(3) *Fate of the cortical hormone in the organism*

The fact that no cortical hormone could be detected in arterial blood, or in blood from the right side of the heart, indicates that the body disposes at great speed of the released hormone. The gastro-intestinal tract, spleen and liver, are not essential for this disappearance, since the result is the same in the eviscerated animal. Even in blood samples which were obtained from the heart several hours after evisceration, cortical activity was absent. If the liver played a predominant role in the inactivation of the hormone, its exclusion from the circulation would lead to accumulation of cortical substance in the general circulation. As no such accumulation was detected, and the hormone is stable in blood, it must be either very rapidly inactivated by the tissues or very speedily excreted by the kidney. In order to decide between the two alternatives, the following experiment was devised: A female cat (2·4 kg.) was anaesthetized with chloralose, and 25 ml. blood were drained from one of its carotids into a tube, containing a little heparin (sample 1). The blood loss was partially replaced by infusing intravenously 15 ml. heparinized blood taken from another chloralose cat which had been bled completely and then discarded. The animal was now eviscerated and both its ovaries and kidneys were excluded from the circulation. Two and a half hours later, 10 mg. heparin were given intravenously, a record of the blood pressure was started and a second sample of 25 ml. blood collected from the carotid. The blood pressure fell from 85 to 6 mm. towards the end of the haemorrhage, but recovered completely when the blood loss was replaced as soon as the sample had been obtained. The aorta was tied distal to the renal arteries, the cava cannulated at the level of the renal veins, the lumbar veins tied at the lateral edge of the suprarenals, the cava ligated above the suprarenals, and the suprarenal effluent drained from the cannulated cava. Twenty-five ml. blood (sample 3) were collected during the next 28 min. and the blood pressure was kept at an average of 62 mm. Hg by slow intravenous infusion of blood (diluted with saline towards the end of the experiment).

The three blood samples were assayed for cortical hormone (Fig. 4, columns 1–3), and their activity was compared with that of saline (column 5) and of a 4 % eucortone solution (column 4). The survival times observed with the two carotid blood samples are identical. Therefore, in the general circulation of the eviscerated, nephrectomized cat, no accumulation of cortical hormone had taken place.

The suprarenal sample, on the other hand, collected in a period of 28 min., contained a considerable amount of hormone (column 3). Its potency was the

same as that of a 4 % solution of eucortone. Assuming that 125 ml. are an adequate estimate of the blood volume of the eviscerated nephrectomized cat, we can infer from that figure and the blood flow through the glands (25 ml. in
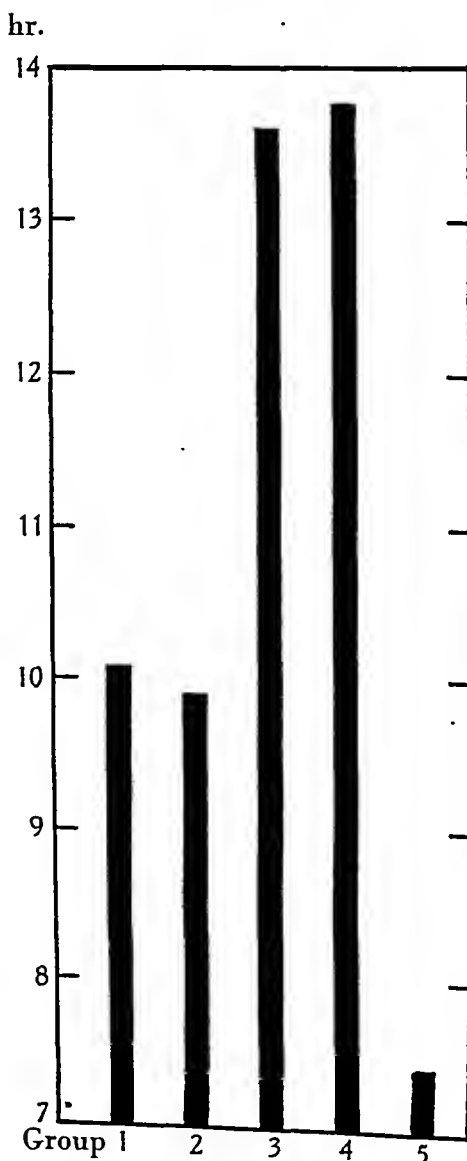
Fig. 4. (Exp. 55, cat.) Ordinate: mean survival time (hours). Number of group at the bottom of each column. Tho rats received at each injectiou: group 1, 0·3 ml. carotid plasma before evisceration; group 2, 0·3 ml. carotid plasma 2¼ hr. after evisceration and uephrectomy; group 3, 0·3 ml. suprareual plasma; group 4, 0·3 ml. 4 % eucortoue; group 5, 0·3 ml. 0·9 % NaCl.

28 min.), that the suprarenals produced in 2½ hr. enough hormone to raise the concentration in the general circulation to the figure found in the suprarenal effluent, provided inactivation had not taken place in the body. As no accumulation whatever occurred, it follows that the substance is destroyed in the tissues and that renal excretion can only play a negligible role in its disposal. This result confirms, for the natural hormone, an observation by Pfiffner, Swingle & Vars [1934], who found only very small amounts of hormone in the urine of a dog injected subcutaneously with a massive dose of cortical extract.

A comparison of the survival time of the saline-treated group (column 5) with those obtained by injection of the two carotid blood samples (columns 1 and 2), shows the slight protection occasionally observed with arterial blood from the cat and mentioned on p. 345. The differences between the survival times, although significant, are so small that there is considerable doubt whether they are due to traces of cortical hormone or to some other constituent of the plasma. As in this cat the ovaries had been tied off at the beginning of the operation, it is unlikely that progesterone was responsible for the protection. Progesterone could be ruled out entirely by another experiment, in which the same phenomenon occurred in a male cat.

DISCUSSION

The foregoing experiments have shown that there is invariably present, in the venous blood leaving the suprarenals, an amount of cortical hormone detectable by biological assay. Its quantity is surprisingly high. The average yield obtained per min. per kg. body weight, by the suprarenal of one side, is equivalent to the amount which can be extracted, by methods used commercially, from 0·6 g. glandular tissue. For a dog of 10 kg. this would represent a daily production by both glands of the equivalent of 17,300 g. suprarenal tissue contained in 230 ml. of a commercial extract.

The discrepancy between the amount of hormone obtainable by chemical treatment of glandular tissue and the quantity present in the venous blood drained from the gland can be interpreted in several ways; either the methods of extraction are very inadequate, or the steroids are not stored in the gland in their potent form but changed into the active principle (or principles) on their release into the circulation. It is not possible at the moment to decide between the two alternatives. If storage of hormone in the cortical tissue is indeed negligible, this fact would distinguish the suprarenal cortex from most other endocrine glands, and particularly from the suprarenal medulla.

Another characteristic feature of cortical activity is the apparently continuous release of the hormone into the circulation; this again is in striking contrast with the rhythmic function of many endocrine glands, the action of which is initiated either by nervous or by chemical stimuli. This observation of permanent activity is, however, so far restricted to the special conditions

(anaesthesia, severe abdominal operation) under which the present experiments were performed. Whether it also holds under normal circumstances cannot be decided before methods have been employed which allow the collection of suprarenal venous blood in the surviving animal.

Comparison of the quantity of cortical hormone secreted by the glands of a medium-sized dog, with the quantities employed for maintaining life in suprarenalectomized animals, discloses another great discrepancy. The figures given by different authors concerning the hormone requirements of suprarenalectomized animals are very variable; some of the differences are obviously due to inequalities in the potency of various extracts; the values, however, are always much smaller than the daily output of hormone into the circulation which, according to the foregoing experiments, corresponds to 230 ml. of a commercial extract for an animal of 10 kg. Eversole, Gaunt & Kendall [1942] state that the maintenance dose for a dog is 1 ml. of cortical extract (75 g. gland), and Cleghorn, Fowler, Wenzel & Clarke [1941] give a similar figure (1·8 ml.) for a dog kept on a diet containing 1 % NaCl. The latter authors add that 7–15 times those amounts are required in the state of collapse; even 27 ml. extract, however, are little compared to 230 ml. According to Nowak [1938], 5 ml. extract daily (approximately 2 ml./kg.) are adequate for a cat, and rats are reported to need between 1 (Eversole *et al.* 1942] and 4·5 ml./kg. [Hartman *et al.* 1931]. It would, therefore, appear that the doses of hormone indispensable to life are far smaller than those available in the animal possessing its suprarenals and required for optimal function of its organs. Some evidence to the effect that the amounts of extracts hitherto used are not adequate for the full restitution of the functions of the body can also be obtained from the treatment of cases of Addison's disease and from functional tests in suprarenalectomized animals. It is well known that human patients, despite the administration of desoxycorticosterone acetate or water-soluble cortical preparations, have a low efficiency of the muscular and circulatory systems. Observations on cortin-treated suprarenalectomized cats and dogs [Hartman, Lewis, Thatcher & Street, 1942] reveal an abnormally high globulin content of the plasma in spite of a clinically satisfactory condition. Furthermore, in suprarenalectomized rats, the cortin requirements for normal work performance far exceed the maintenance dose; even with very large amounts restitution of function is not complete [Ingle, 1936]. The possibility has, of course, to be borne in mind that the extracts used are not only given in too small a quantity, but might be qualitatively different from the natural hormone.

SUMMARY

1. Experience is reported with Selye & Schenker's method for the assay of cortical hormone (treatment with cortical extract of young, suprarenalectomized rats exposed to low temperature). The response (mean survival time)

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was found to be proportional to the logarithm of the dose. The accuracy of the test is inferior to that of the assay on drakes; its sensitivity, however, is about 25 times higher.

2. With the aid of this assay, estimation was made of the cortical hormone in the venous effluent of the suprarenal gland of the dog, the cat, the goat, the rabbit and the pig. Considerable cortical activity was invariably found in the venous blood collected from the suprarenals. It was absent in arterial blood and in blood taken from the right side of the heart.

3. The potency of suprarenal blood was compared with that of a commercial extract ('eucortone', Allen and Hanburys), of which 1 ml. is equivalent to 75 g. gland. The potency of the blood may then be expressed by the weight of suprarenal tissue which has to be extracted in order to obtain a solution of the same activity. The average output of one suprarenal gland, per min. per kg. body weight, was equivalent to 0·6 g. of suprarenal tissue. Its range lay between 0·26 and 2·0 g. tissue. The order of magnitude was the same in the five species used. The potency of 1 ml. suprarenal plasma was at least several times, occasionally 10–12 times, as high as the activity obtained by extraction of 1 g. gland.

4. The figure of 0·6 g. suprarenal tissue per min. per kg. amounts to a daily output, by both suprarenals of a 10 kg. dog, of 17,300 g. gland contained in 230 ml. of a commercial extract. This figure is probably an underestimate. It follows that either our present methods of extraction are inadequate, or the suprarenal cortex does not store its principle (or principles) in the active form.

5. As far as can be detected by the available methods, the minute-output by the suprarenal cortex is not affected by changes in blood pressure or blood flow.

6. Cortical hormone is rapidly inactivated in the tissues. Liver, spleen and gastro-intestinal tract are not essential for this process. Neither does the excretion by the kidney play an important part in the disposal of cortical hormone.

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METABOLISM OF PHOSPHATE AND CARBOHYDRATE IN EXTRACTS OF HUMAN MUSCLE AND BRAIN

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An outstanding factor in the intermediary metabolism of muscle is the part played by phosphate. The immediate source of energy for muscle contraction is not, as once thought, the breakdown of glycogen to lactic acid, but is most probably the splitting of phosphate from adenosinetriphosphate (reaction A—see below). This appearance of inorganic phosphate is brought about by an enzyme intimately associated with myosin, the contractile protein of muscle. The adenosinetriphosphate, which is present only in small amounts, is reconstituted in two ways:

(a) Phosphate is transferred from compounds formed during the breakdown of glycogen into lactic acid. The conversion of the large glycogen molecule into a 3-carbon compound involves a chain of interlinked reactions. Glycogen simultaneously takes up phosphate and breaks down into the 6-carbon compound hexosemonophosphate (reaction B). This receives a second phosphate group to become hexosediphosphate, which in turn splits into the 3-carbon compound triosephosphate (reaction C). Like hexosemonophosphate, triosephosphate undergoes further phosphorylation. It becomes triosediphosphate, which passes by various stages into lactic acid. At two of these, a phosphate group is yielded up for the reconstitution of adenosinetriphosphate.

(b) The more available source of phosphate for the resynthesis of adenosinetriphosphate is, however, creatine phosphate ('phosphagen'), for whereas glycogen must first undergo various transformations, phosphagen donates its phosphate directly. During this reaction (D), free creatine appears, and the phosphagen level falls. The process is reversible, however, so that adenosinetriphosphate can phosphorylate free creatine. Hence, as adenosinetriphosphate can be resynthesized through glycogen breakdown, phosphate received during lactic acid formation can be used to replenish the phosphagen store. The latter, the most readily tapped reservoir of energy in muscle, can thus be refilled at the expense of glycogen breakdown.

Our knowledge of these reactions is based on work with *animal* muscle. Some have been shown to occur in tissues other than muscle, as well as in bacteria, yeast and higher plants. However, it has not, to our knowledge, been established that these processes occur in *man*.

As a first step we have selected the following four reactions, the significance of which has been explained above:

A. Adenosinetriphosphate → adenosinediphosphate + phosphate.
B. Glycogen + phosphate → hexosemonophosphate.
C. Hexosediphosphate ⇌ 2 triosephosphate.
D. (1) Adenosinetriphosphate + creatine ⇌ adenosinediphosphate + creatine phosphate.
    (2) Adenosinediphosphate + creatine ⇌ adenylic acid + creatine phosphate.

They represent the release (A) and uptake (B) of inorganic phosphate, and the reversible transfer of phosphate from adenosinetriphosphate to creatine (D); also the breakdown of a polysaccharide into a 6-carbon compound (B), and of the 6-carbon chain into the 3-carbon chain, the skeleton of lactic acid (C).

We have found that all these reactions take place in cell-free extracts of human muscle.

METHODS

1–3 g. specimens of normal human voluntary muscle were obtained from fourteen patients, during operations under spinal anaesthesia for various surgical conditions such as inguinal hernia, carcinoma of the rectum, retroversion of the uterus, etc. Specimens of the following muscles were obtained: rectus abdominis, obliquus abdominis, pyramidalis and cremaster. All four reactions were demonstrated in at least two specimens. We also obtained three samples of normal brain tissue, mainly cortex, from the frontal lobe, two during prefrontal leucotomy, and one during the removal of an intracranial tumour. Extracts of these were used to show the formation of triosephosphate (C) and the phosphorylation of creatine (D). Only one uterus sufficiently free from fibroid degeneration was secured. Its muscle was used to demonstrate triosephosphate formation (C).

Aqueous cell-free extracts were prepared according to Meyerhof [1926], the various tissues, whether human or animal, being treated alike. Immediately after excision they were cooled by ice. They were then without delay freed as completely as possible from adherent blood and fat, weighed, cut finely with scissors, and ground with sand in a cooled mortar. They were next extracted with 1·5 vol. of ice-cold water for 20 min. in the refrigerator, with occasional stirring, and centrifuged for 10 min. at 3000 r.p.m. The supernatant fluid was left for 30 min. at room temperature, and then stored in the refrigerator, with toluene as preservative.

We prepared myosin by the method of Greenstein and Edsall, following the directions of Bailey [1942] although working on a much smaller scale. For use, the myosin gel was dissolved in 0·5 M KCl, and therewith brought to the volume of the original muscle sample.

ANALYTICAL METHODS

The reactions were stopped by deproteinization with trichloroacetic acid. Inorganic phosphate was determined by Lohmann & Jendrassik's [1926] modification of the Fiske & Subbarow method

a Hilger Absorptiometer being used for the colorimetry. The various phosphate esters were determined as follows:

Adenosinetriphosphate. As inorganic phosphate (corresponding to two of the three phosphate groups) released by 7 min. hydrolysis with N HCl at 100° ('7 min. P_2O_5') [Lohmann, 1928].

Hexosemonophosphate. As inorganic phosphate released on ashing the isolated water-soluble barium salts [Cori & Cori, 1931].

Triosephosphate. As inorganic phosphate released by 20 min. hydrolysis with N NaOH at room temperature [Meyerhof & Lohmann, 1934].

Creatinephosphate. As the difference between the 'inorganic phosphate' as usually determined and the true inorganic phosphate precipitated by Mathison's reagent [Lohmann, 1928].

All data refer to human material unless otherwise indicated.

A. *Breakdown of adenosinetriphosphate.* With myosin in dilute solution one phosphate group only is released. With higher concentrations, contamination by adenosinediphosphatase may be sufficient to cause release of more than one phosphate group, i.e. of more than 50 % of the '7 min. P_2O_5' [Bailey, 1942]. This is shown in Table 1.

TABLE 1. Each sample contained 1 ml. 0·1M glycine-NaOH buffer (pH 9·2 at 40°), 0·05 ml. 0·18M CaCl$_2$, and 0·5 ml. adenosinetriphosphate solution containing 0·61 mg. 7 min. P_2O_5. Temp. 37°.

Myosin solution (ml.)	Time (min.)	% of 7 min. P_2O_5 released
0·1	15	41
0·1	30	49
0·1 (heated to 100°)	30	2
0·3	30	75

Table 2 shows the activation of myosin by Ca^{++} [Needham, 1942; Bailey, 1942], and its reversal by Mg^{++} [Greville & Lehmann, 1943], an antagonism which may have a bearing on muscle excitability.

TABLE 2. In each sample, 0·1 ml. myosin solution, 1 ml. buffer as before, 0·5 ml. adenosinetriphosphate solution containing 0·69 mg. 7 min. P_2O_5; total vol. 1·9 ml. 10 min. at 37°.

CaCl$_2$ or MgCl$_2$ added	% of 7 min. P_2O_5 released
None	3
0·01 M MgCl$_2$	4·5
0·005 M CaCl$_2$	42·5
0·005 M CaCl$_2$ + 0·01 M MgCl$_2$	9

B. *Glycogen phosphorylation.* This reaction is a 'direct phosphorylysis', and does not involve, as was long believed, an initial hydrolysis of glycogen to glucose. Hence in absence of amylase, the inorganic phosphate which disappears is a measure of the glycogen breakdown. In our experiments the

TABLE 3. In each sample, 0·3 ml. muscle extract containing 0·22 mg. inorganic P_2O_5, 15 mg. glycogen, 0·03 mg. Mg^{++} as MgCl$_2$, 2·04 mg. P_2O_5 as buffer pH 7·4; total vol. 1 ml. NaF was added to prevent phosphatase action on the esters formed [Ostern, Herbert & Holmes, 1939], and toluene as preservative.

Temp.	Time (hr.)	NaF	% phosphate esterified
Room	12	—	57
Room	12	0·01 M	70
37°	1	—	38
37°	1	0·02 M	46

former was occasionally controlled by determination of the hexosemonophosphate formation.

C. *Equilibrium between hexosediphosphate and triosephosphate*. This equilibrium is dependent on the temperature and the total concentration of the esters. The higher the former and the lower the latter, the more it shifts in favour of triosephosphate [Meyerhof & Lohmann, 1934]. Starting with a hexosediphosphate concentration of 0·33 mg. P_2O_5 per ml., the conversion to triosephosphate at equilibrium is 50 % at 37° and 15 % at 0°.

TABLE 4. The extracts formed 1/12 of the total vol. Hexosediphosphate, 0·33 mg.
P_2O_5 per ml., pH 7·4; phosphate buffer, 0·0075 M, pH 7·4. 37°.

	% conversion to triosephosphate in		
Source of extract	1 min.	5 min.	15 min.
Voluntary muscle: Human	19	50	50
Rabbit	48	48	45
Cerebral cortex: Human	11	29	50
Rabbit	11	30	46
Guinea-pig	12	37	48
Uterus muscle: Human	5	9	20
Rabbit	3	11	26

Table 5 shows the resynthesis of hexosediphosphate from triosephosphate when the equilibrium shifts on alteration of temperature.

TABLE 5. Muscle extract, reaction mixture as in Table 4.

	% triosephosphate
60 min. at 0°	16
10 min. at 37°	52
10 min. at 37°, followed by 60 min. at 0°	16

D. *Phosphate transfer between adenosinetriphosphate and creatine.* Reactions D 1 and D 2 are interdependent. The equilibrium point of each depends on the pH; the more alkaline the mixture the more creatinephosphate present at equilibrium [Lehmann, 1935, 1936]. It will be seen (Table 6 (a)) that creatine phosphate formed at an alkaline reaction breaks down again at neutrality with reconstitution of adenosinetriphosphate.

TABLE 6. (a) In each sample, 0·3 ml. *muscle* extract (stored 17 days in refrigerator), 0·1 ml.
0·1 M Na_2HPO_4, 1 ml. 0·1 M glycine buffer (pH 9·7 at 18°), 10 mg. creatine, adenosinetriphosphate 1·16 mg. 7 min. P_2O_5. The whole was diluted to 3 ml.; it could be brought to ca. pH 7 by adding 0·35 ml. 0·1 N HCl, and returned to the original pH with 0·35 ml. 0·1 N NaOH. 17°.
(b) 0·13 ml. *brain* extract, other additions as in (a), total vol. 2·1 ml. 18°.

	% of 7 min. P_2O_5 transferred to creatine
(a) 15 min. at pH 9·7	78
15 min. at pH 9·7, followed by 20 min. at pH 7	27
15 min. at pH 9·7, followed by 20 min. at pH 7, then by 25 min. at pH 9·7	72
(b) 30 min. at pH 9·7	29

Summary

Reactions involving phosphate esters have been shown to occur in extracts of human voluntary muscle, brain cortex and uterus muscle.

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TABLE 6. (a) In each sample, 0·3 ml. *muscle* extract (stored 17 days in refrigerator), 0·1 ml. 0·1M Na$_2$HPO$_4$, 1 ml. 0·1M glycine buffer (pH 9·7 at 18°), 10 mg. creatine, adenosinetriphosphate 1·16 mg. 7 min. P_2O_5. The whole was diluted to 3 ml.; it could be brought to *ca.* pH 7 by adding 0·35 ml. 0·1N HCl, and returned to the original pH with 0·35 ml. 0·1N NaOH. 17°.

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15 min. at pH 9·7, followed by 20 min. at pH 7, then by 25 min. at pH 9·7	72
(b) 30 min. at pH 9·7	29

results of these experiments Hammarsten & Jorpes found support for their theory that the alkali in the pancreatic juice is stored in the gland and discharged from these supplies during secretion.

Ball [1930] took as his starting point the assumption that if the alkali in the juice on secretion comes direct from the blood, then changes in the bicarbonate content of the plasma and in the concentration of each individual cation in the blood ought immediately to affect the concentration of the ions concerned in the pancreatic juice. Ball's experiments were made on dogs, the secretion being set in motion by injections of secretin. He administered hydrochloric acid intravenously, and produced a comparatively high reduction in the bicarbonate content of the blood; this was answered by a slight fall in the bicarbonate content of the pancreatic juice. He raised the bicarbonate content of the blood by an injection of carbonate and the bicarbonate radical in the pancreatic juice rose. After this procedure also, a relatively large increase in the bicarbonate content of the blood was obtained but only a slight increase in that of the pancreatic juice. When he increased the sodium and potassium in the blood, these ions rose at once in the pancreatic juice. Thus Ball found that alterations of the bicarbonate radical, the potassium, and sodium in the blood were immediately followed by similar changes of these ions in the pancreatic juice; the pancreas is therefore readily permeable to these ions. He considered he had proved by these tests that the alkalinity of the pancreatic juice originates directly from the blood. Objections may be raised against Ball's experimental method; the alterations in the equilibrium between acids and bases which he produced in the blood of his experimental animals are large, and fall outside the physiological limits. Furthermore, the changes in the bicarbonate content of the pancreatic juice were small in relation to the great changes in the alkali reserve of the serum. In addition, his results are made more difficult to interpret through the fact that, as he himself demonstrated, the bicarbonate content in the pancreatic juice varies according to the secretion rate. When the rate increases the bicarbonate content in the juice increases. His experiments on the effects on the pancreatic juice of alterations in the blood's content of potassium and sodium show, however, that an alteration in the concentration of these ions in the plasma is accompanied by a corresponding change in the pancreatic juice.

As was proven by Montgomery, Sheline & Chaikoff [1941], radioactive sodium given intravenously appears in the pancreatic juice within 3 min. This, of course, supports the theory that the alkali in the pancreatic juice originates directly from the plasma.

In the investigation to be described in this paper, Hammarsten & Jorpes's theory has been subjected to further experimentation.

If the alkali in the pancreatic juice is delivered from a supply of alkali in the gland, one would expect, as Hammarsten & Jorpes pointed out, that this

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THE ALKALI IN THE PANCREATIC SECRETION*

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There has been some discussion as to whether the alkali in the pancreatic juice is delivered from reservoirs in the gland or whether it is taken direct from the blood during the pancreatic secretion. In 1928, Hammarsten & Jorpes advanced the theory that the alkali is stored in the gland as nucleic acid alkali and that this alkali bound to the nucleic acids is first taken into use when the secretion takes place. Ball [1930], after having observed that changes in the sodium and potassium content of the blood are immediately followed by changes of these ions in the pancreatic juice, concluded that the alkali in the juice is delivered direct from the blood during the secretion.

If the theory advanced by Hammarsten & Jorpes is correct, and the charging of the gland with alkali proceeds at a slower rate than the discharging, with a maximum secretion, their assumption ought to be experimentally verifiable. These investigators, by means of repeated injections of secretin, caused their experimental animals (cats) to secrete maximum amounts of pancreatic juice. The animals' stomachs had been removed in order to exclude all possibility of a simultaneous secretion of gastric juice. Control cats operated upon in the same way and at the same time were used as comparative material. The first experiments were made with a view to finding out whether the secreted amounts of alkali produced any change in the alkali reserve in the blood of the experimental animals, but no such change could be demonstrated. It was also thought that if the pancreas could be worn out by powerful and sustained secretion the accumulation of acid radicals in the gland which, according to their theory, ought to take place, should bring about a reduction in the alkali reserve in the gland; such a reduction when sufficiently advanced, should cause an increase in the degree of acidity in the gland. Hammarsten & Jorpes produced an extract from the finely cut-up gland and made pH determinations on the extract. They observed that the degree of acidity in the extract from a normal pancreas was in all cases lower than that in the extract from the secreting pancreas. From the

normal cats. The alkali reserve of the pancreas therefore does not change during secretion. The experiments point consequently to the fact that the alkali released during the secretion of pancreatic juice is not stored in the gland as was suggested by Hammarsten & Jorpes. The amounts of alkali collected during these secretion experiments exceeded many times over the alkali which, according to the last-mentioned investigators, could be bound by the nucleic acids in the gland of a cat (3 c.c. of $0.1 N$ alkali).

Another way of testing the secretion theory under investigation might be to determine the alkali metals in the gland when at rest and after intensive secretion. I carried out a few experiments of this type.

The experimental animals were cats, and these were operated upon and treated in the same manner as for the preceding experiments. Immediately after the close of the experiment the pancreas was excised and freed as thoroughly as possible from fat and blood. It was divided into two parts and these were weighed and incinerated with perchloric acid. The surplus perchloric acid was evaporated and the ash dissolved in distilled water to make up a certain volume, usually 25 c.c. From the solution the potassium was determined by the method used by Cullen & Wilkins [1933], the sodium by the method of Butler & Tuthill [1931], and of Cullen & Wilkins, and the calcium by Lundegårdh's [1929] method. Table 3 shows the findings.

TABLE 3. Determinations of the alkali in the pancreas

Exp. time min.	Collected pancreatic juice c.c. of $0.1 N$ NaOH	K m.equiv./kg.	Na m.equiv./kg.	Ca m.equiv./kg.	K + Na + Ca m.equiv./kg.
180	27.5	112	61	3	176
25	4.4	107	68	3	178
		113	65	3	181
—	0	107	72	3	182
		111	70	3	184

(Alkali in the gland tissue spans columns K, Na, Ca)

Not even in these experiments could any difference be observed between the pancreas at rest and after secretion. The results support the observations made during the determinations on the alkali reserve.

SUMMARY

1. The alkali reserve of the pancreas of cats after profuse secretion of pancreatic juice is not different from that of the resting gland.

2. The potassium, sodium and calcium content of the pancreas of cats does not change as a result of secretion.

3. The origin of the alkali of pancreatic juice is discussed and it is concluded that it is obtained directly from the blood during secretion.

alkali reserve would decrease during intensive secretion. In order to test whether this really occurs the following experiments were carried out.

Cats were fasted for 24 hr. and anaesthetized with urethane. The duodenal papilla was laid free and 20 doses of secretin [Wilander & Ågren, 1932] were given intravenously every 15 min. for 2–3 hr. By this means a continuous and powerful secretion of pancreatic juice was obtained. The secretin compound had very kindly been placed at my disposal by Ågren & Wilander [1933], and by Hammarsten and his co-workers [1933]. In some of the tests the secreted juice was collected in the manner suggested by Wilander & Ågren and the alkali titrated. Immediately after the conclusion of the experiment the cat was killed and the pancreas removed and freed from fat as completely as possible. The gland was minced by cutting slips 10μ thick on the freezing microtome, and the process was then repeated. Part of it was transferred to a weighing flask and weighed (2–3 g.). Distilled water (as a rule about 5 c.c.), a few drops of octyl alcohol, and a number of glass beads were added and the flask was energetically shaken. A homogeneous suspension of the gland in water was thus produced. The suspension was then saturated at 38° C. with a mixture of air and carbon dioxide having a carbon dioxide pressure of 40 mm. The alkali reserve in the gland suspension was then determined immediately by Van Slyke's manometric method, on 1 c.c. of the suspension [Peters & Van Slyke, 1932]. Control animals were treated in exactly the same manner, except that they received no secretin injections. The values obtained will be seen in Tables 1 and 2.

In these experiments the stomach was not removed; this did not seem necessary in view of the hypothesis on which the tests were based. The pylorus was tamponated.

TABLE 1. Pancreatic secretion set free by secretin

Cat no.	Alkali reserve mM. BHCO₃/kg.			Mean mM. BHCO₃/kg.	Collected alkali c.c. of $0.1N$ solution
2	18·7	18·5		18·6	6·5
4	21·8	18·7		20·3	10·2
5	18·4	18·2		18·3	2·8
7	18·4			18·4	7·0
8	19·4	18·7		19·1	25
10	20·8	19·9	19·1	19·9	22

TABLE 2. Normal cats

Cat no.	Alkali reserve mM. BHCO₃/kg.			Mean mM. BHCO₃/kg.
1	18·9	19·0		19·0
3	18·8	18·3		18·6
6	18·9			18·9
9	20·6	20·6	20·1	20·4

The mean figure for the alkali reserve was thus 19·1 mM. of BHCO₃/kg. of gland for the secreting cats, and 19·2 mM. of BHCO₃/kg. of gland for the

A COMPARATIVE BIOLOGICAL ASSAY OF ACTIVITY IN SIMPLE SOLUTIONS OF ADRENALINE

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When a sample of adrenaline is to be tested to see if it has the full activity of the pure substance, the biological assay is usually performed using the vaso-pressor action in a spinal cat or dog, or by its action on the isolated rabbit intestine. Isolated frog hearts prepared by Straub's method have also been utilized [Gaddum & Kwiatkowski, 1939], and estimates by this method were found to agree with colorimetric determinations [Shaw, 1938]. Frogs are more easily obtained in sufficient quantities than cats or dogs at the present time, and so perfusion of the frog heart was studied as a means of assaying activity in simple pharmaceutical solutions of adrenaline.

METHOD

The isolated frog heart was perfused by a modified Symes's method. Having exposed the heart free from pericardium and having ligatured the anterior venae cavae, a mammalian venous cannula, full of frog Ringer's solution, was inserted into the posterior vena cava. The heart was then removed from the animal and the movements recorded on a kymograph by means of a thread and a long isotonic lever. The cannula was connected to the perfusion apparatus (see Fig. 1), which was supported on one retort stand. The frog Ringer's solution in reservoir *A* was maintained at a constant level (as shown by an arrow), the adrenaline dilution in bottle *B* also being at this level before perfusion. The heights of the two siphon tubes were equal, being about 5 in. above the venous cannula. Results were consistent using this apparatus, although Mariotte bottles could be used to maintain a constant pressure of perfusion.

PERFORMANCE OF THE ASSAY

A dilution of the standard adrenaline solution was placed in bottle *B*, and tap *B* was opened so that this solution siphoned over by blowing down outlet tube *B*. Using a slow drum, a short tracing was taken to show the normal beat, and then the three-way capillary tap *C* was turned to allow perfusion of the

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aliquot portions were removed and made up to 100 ml. with Ringer's solution in flask 3. This was the perfusion fluid, and it was used immediately and never more than once, as over 30 % of the activity is lost in 5 min. On most hearts, the standard gave an adequate response in a concentration of 1/100,000 (that is, 1/100,000,000 of adrenaline).

RESULTS

Over forty samples of simple adrenaline solutions have been assayed. For each experiment, the standard solution was freshly prepared unheated liquor adrenaline of the British Pharmacopoeia (1/1000). Fig. 2 shows a comparison between this standard (S) and a test solution (T). From these tracings, (a) 1/150,000 of test was stronger than 1/200,000 of standard. or test was

T S   S T   T   S   S T
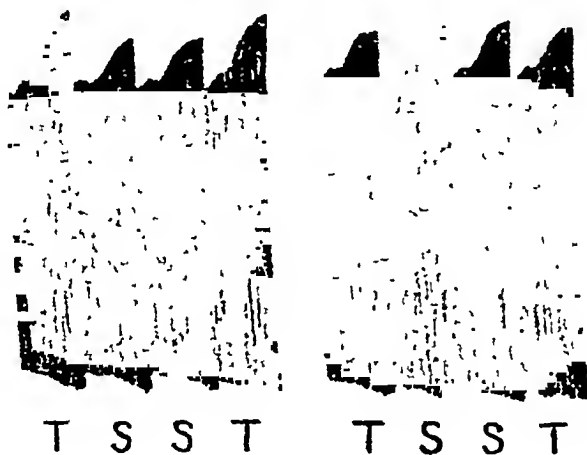
Fig. 2. Effects of standard (S) 1/200,000 compared with test (T) 1/150,000 and 1/200,000. Each perfusion lasted for 1½ min. only.

stronger than 150,000/200,000 = 0·75 of standard; (b) 1/200,000 of test was weaker than 1/200,000 of standard, or test was weaker than standard. Hence. the approximate strength of the test solution was 87·5 % of the standard, that is, 1/1143 of adrenaline. The comparison was then repeated using different doses of the test solution so as to obtain closer limits.

The results obtained using varying dilutions of the freshly prepared standard are recorded in Table 1. Freshly boiled and cooled distilled water was used for preparing the dilutions, which were carried out by an independent worker. The standard error of the test, calculated by the method of Gaddum [1938] using the true values of the 'unknown' dilutions, was 2·17 %. The limits of error ($P = 0.99$), therefore, are 100 ± 5.59 %. It is recognized that differences of 5–6 % can be accurately measured by the cat method, so that the frog-heart method is just as accurate.

adrenaline solution in place of the Ringer's solution. This perfusion lasted exactly 1½ or 2 min., after which the lever was removed from the drum. The drum was stopped, and tap C turned to allow the Ringer's solution to reperfuse. The contents of bottle B and of the siphon tube B were removed, and the liquid remaining in the tube between tap C and tap B was washed out with Ringer's solution by turning tap C and opening tap B. The dilution of the test adrenaline solution was then prepared, and this took exactly 4 min. During this period the heart beat had nearly always returned to normal. The drum was started, and the test solution perfused for the same fixed time, at the end of which it was replaced by Ringer's solution. The test adrenaline was repeated using the same dose, and, finally, the standard so that a group of four
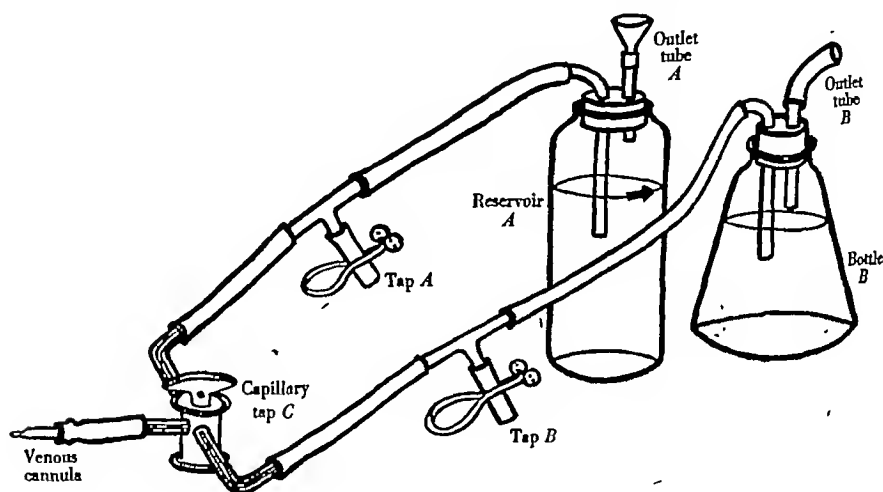
Fig. 1. The perfusion apparatus. The retort stand has been omitted; it supports reservoir A, bottle B and the three-way capillary tap C.

results was obtained. It was found that the response to a given dose was more constant in the region of the maximum response than elsewhere, and so solutions were compared in exactly the same manner as that used for the official assay of pituitary (posterior lobe) extract by using doses which just produced submaximal contractions. Equality of response was useful only as confirmatory evidence.

The most accurate procedure for obtaining dilutions was to have the adrenaline solutions supplied in 2 ml. ampoules. Each ampoule was used for one response only, and dilutions were always carried out using one complete set of pipettes and three 100 ml. volumetric flasks. One ml. of the solution in the ampoule was diluted with 99 ml. of freshly prepared boiled and cooled distilled water in flask 1; of this dilution, 1 ml. was removed and diluted with 99 ml. of freshly boiled and cooled distilled water in flask 2; of this dilution,

Summary

A biological assay of adrenaline solutions by the effect on isolated perfused frog hearts is described. The method has the virtue of using the same tissue for the standardization, so allowing direct comparison between the standard and test solutions with the opportunity of interchange so as to make comparisons and contrasts. Winter male frogs provide the best test objects. The limits of error ($P = 0.99$) of the test were estimated as 100 ± 5.59 %.

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TABLE 1. The estimation of unknown dilutions of the standard adrenaline solution

Dilution of standard (as %)	Values found	Mean of four results	Individual percentage deviations of the true result
90	90·9, 91·7, 91·7, 90·9	91·3	1·0, 1·9, 1·9, 1·0
80	80·1, 80·1, 80·1, 83·3	80·9	0·1, 0·1, 0·1, 4·1
70	71·7, 71·4, 69·0, 71·7	70·9	2·4, 2·0, 1·4, 2·4
60	60·7, 59·0, 60·7, 60·7	60·3	1·2, 1·6, 1·2, 1·2
50	50·0, 51·3, 51·3, 48·8	50·4	0·0, 2·6, 2·6, 2·4
40	40·8, 38·5, 39·3, 40·8	39·9	2·0, 3·7, 1·7, 2·0
30	30·3, 29·6, 29·9, 30·3	30·0	1·0, 1·3, 0·3, 1·0

Comparison with the rabbit-intestine method

Three dilutions were assayed by the frog-heart method and then by the rabbit-intestine method. The results are recorded in Table 2.

TABLE 2. Estimation of adrenaline solutions by the two methods

Dilution of standard (as %)	Frog-heart method (using a concentration of $1/10^8$ of adrenaline)		Rabbit-ileum method (using a concentration of $1/5 \times 10^7$ of adrenaline)	
	Value obtained (as %)	Percentage deviation	Value obtained (as %)	Percentage deviation
90	90·9	1·0	91·4	1·6
60	60·7	1·2	59·0	1·6
30	29·5	1·7	28·6	4·7

These results show good agreement. The frog-heart method was not only slightly more sensitive than the rabbit-gut method (as a lower concentration of the standard adrenaline was sufficient), but it also showed lower percentage deviations. The sensitivity could be still further increased by cocainization; when a concentration of 1/333,333,333 of adrenaline was perfused through the frog heart alone, no stimulatory effect was observed, but when perfused with 1/200,000 of cocaine hydrochloride, a measurable response was shown.

DISCUSSION

Repeated perfusion of the same dose of standard adrenaline produced similar responses, but after 4 or 5 hr. the response diminished gradually. This did not upset the assay, the result being based on groups of four tracings, and these were reproducible. It is important to keep a fixed time interval between doses, and this, of course, will depend on the output of the heart. Large winter male frogs have been used throughout. Results were not so consistent with summer frogs, the hearts of which needed larger doses and were always useless after about 3 hr. The heart-beat was about 30–40 per min. in nearly every experiment; if the ligature had been placed on auricular muscle, the beat was much reduced and the preparation was useless for the test.

ANAPHYLAXIS TO SERUM PROTEINS
IN THE GUINEA-PIG

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PART 1. ABSORPTION FROM THE INTESTINE

Since the pioneer work of Van Slyke & Meyer [1912] demonstrated the ready absorption of amino acids from the intestine, experiments on the absorption of undigested protein have been almost entirely lacking. It is assumed that during the course of digestion protein is absorbed in the form of amino acids, and this assumption is based on the ample provision of the appropriate enzymes in the gut. It is not known whether individual proteins are prevented from passing the epithelium without previous hydrolysis, on account of the large size of the molecule or some special property of the cells which rejects foreign protein. To explain certain allergic phenomena in man the absorption of protein in small amounts is conceded, but this is without direct proof, and in any case is usually regarded as a pathological process in certain individuals. There seems no reason why molecular size should of itself limit the absorption of unchanged protein from the intestine.

The present work was undertaken to determine whether horse serum albumin and serum globulin, two proteins which differ markedly in molecular weight and which, as Svedberg & Sjögren [1928] have shown, may be isolated homogeneous as regards particle size, can be absorbed from the small intestine; and if so, whether molecular size has an appreciable influence, enabling albumin to be absorbed more readily than globulin. Since it was not to be expected that these proteins would be absorbed in large amount, the ideal method of approach seemed to be by the application of the anaphylaxis reaction. This method has been used with success by Hettwer & Kriz [1925] and by Hettwer & Kriz-Hettwer [1926] in studying the absorption of blood serum from the small intestine of the guinea-pig sensitized by serum. These workers injected serum into loops of intestine of anaesthetized animals and noted whether symptoms of anaphylactic shock occurred. Desensitization was also tested by subsequent intraperitoneal injection of serum. We have carried out upwards of two hundred experiments on anaphylactic shock in guinea-pigs,

Dale & Hartley [1916] had·shown that a longer time is needed for albumin sensitization, 4–5 weeks was allowed for the latter. The animals were weighed just before operation, and we have worked always to the nearest 20 g. body weight; an imaginary standard animal of 300 g. or 15 'units' has been adopted, and the weights of protein necessary for injection, and many calculations, have been made on this basis. Only for publication are the weights of protein recorded as per 100 g. body weight.

When sensitization should have been effected, the animal was anaesthetized with ether, the abdomen was opened, and two ligatures were placed on one uterine horn. Two threads were inserted in the muscle between the ligatures and the piece of muscle was removed and set up in the bath, the contractions being recorded on smoked paper. Latterly, the two ligatures were dispensed with; some little time was saved and no significant amount of blood was lost. After the first horn had been removed, the protein was injected into the animal. The wound was then sewn up and the animal was allowed to recover from the anaesthetic. After the desired interval a portion of the second horn was removed, usually after killing the animal by a blow on the head, but often under anaesthesia in the living animal. It has been impossible to detect any interfering action of the ether throughout the experiments. Except where otherwise stated, in testing the first and second horns for sensitivity 0·1 c.c. of whole horse serum was added to the bath, whether the animals were sensitized to albumin, globulin, or serum; this was more convenient and economical of material. The muscle bath contained 120 c.c. of Ringer-Locke's solution: temperature was maintained at about 37° C. by the burner and copper rod figured by Dale & Laidlaw [1912]. After several hundred experiments in which Ringer solutions were used with the normal and half the normal quantities of calcium at different times [see Dale, 1912], a preference for the latter was established and this has been used throughout the latter half of the work.

RESULTS

Absorption of globulin and albumin from the intestine

The highest possible level in the small intestine was chosen as the site of injection, since during life there would be little chance of undigested protein reaching the lower levels, owing to the ample provision of digestive enzymes. If it could be shown that protein could pass through the upper part of the small intestine when introduced artificially, then it might be conceded that this process may occur· under more normal conditions. The protein was 'dissolved' in normal saline, no attempt being made to bring the globulin into more complete solution by means of alkali. In these and subsequent experiments no volume of fluid greater than 1·0 c.c. was injected into intestine or blood vessels unless otherwise stated. Injection was made into the first

giving the reacting dose by intracardiac injection into the unanaesthetized animal, and concluded that the symptoms of shock shown by the animals are somewhat unreliable. If death occurs in a few minutes there can be little doubt, but short of this, misleading conclusions may be reached in many cases.

For the quantitative experiments which were planned in the present work it seemed that the sensitized animal could be used to greater advantage by employing females only, removing one uterine horn and then injecting the protein. While absorption was taking place the first horn could be tested for sensitivity by the method of Dale [1912]. The second horn could then be removed after an appropriate interval and similarly tested. This method has the disadvantage that only females can be used, but the much greater advantages that the initial sensitivity or otherwise of the animal is established, and that the response of the isolated uterus is more reliable than are the symptoms of the whole animal.

MATERIAL AND METHODS

Horse serum albumin was crystallized by the method of Young [1922], twice washed and twice recrystallized by the method of Adair & Robinson [1930]. The crystals of albumin were drained as completely as possible on porous tiles, dried over sulphuric acid and ground to powder. The ammonium sulphate content of the albumin preparations varied from 11 to 15%.

Whole globulin was used, since the work of Svedberg & Sjögren [1930] has shown that euglobulin and pseudoglobulin are very unhomogeneous in solution and are artefacts derived from the parent globulin. The globulin was prepared by precipitation from serum by the addition of an equal volume of saturated ammonium sulphate. After filtration for 5-6 hr. the globulin precipitate was dissolved in a volume of water equal to half that of the serum used. An equal volume of ammonium sulphate was added and filtration was carried out after 5-6 hr. The globulin was precipitated and redissolved four times in all, then the precipitate was drained on tiles, dried and ground. The ammonium sulphate content of some of the globulin preparations was much higher than that of the albumin, and to avoid large differences which would involve troublesome calculations in dosage, all globulin preparations were rejected which had an ammonium sulphate content higher than 20%. Those preparations which were used contained 12–20% ammonium sulphate. When allowance for the salt content had to be made, the ammonium sulphate content of both albumin and globulin was taken to be 15%, a sufficiently close approximation. All figures for the albumin and globulin dosage given are the weights of the dry powder, and no account of the ammonium sulphate is taken unless specifically stated. Sensitization was by intraperitoneal injection of 2 mg. albumin or globulin in 0.1 c.c. 0.9% NaCl. An interval of 2–4 weeks, usually 19–21 days, was allowed in the case of globulin, but, since

desensitization was effected when the dose of protein was above 4·0 mg./100 g. body weight. No symptoms of anaphylactic shock were observed in any experiment.

TABLE 3. Animals sensitized by albumin. Albumin injected into the portal blood

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
0·5	+ − −	4·0	+ +
1·0	− − −	5·0	− −
2·0	− − +	6·0	− − − − − −

Desensitization of the globulin-sensitized animal by globulin injected into the portal circulation

This series was started, as in the case of the corresponding series with albumin, by injecting a small amount of globulin into the mesenteric vein and gradually increasing the dose in succeeding experiments. In these animals severe shock, sufficient to cause death in 3–5 min. after injection of the protein, was met with for the first time. After the death of three animals, as recorded in Table 4, it was clear that though the whole dose might be given in one injection without a fatal result, there was a grave risk of death, and attempts were made to reduce this risk by spreading the dose over two or three injections. This was first done by giving a small amount of protein after removing the first horn, waiting 5 min., and then injecting the second. The third injection, in which the remaining protein was given to bring the whole amount to the desired total, was given 1½–2 hr. later after anaesthetizing the animal and reopening the abdomen. Guinea-pigs stand a second operation very well, and even a third if necessary. When it was thought that an initial dose of 1·0 mg./100 g. could be safely given without shock, two animals given this amount died with typical symptoms, gasping respiration and passage of urine, 3 min. after the injection. The results to this stage are given in Table 4,

TABLE 4. Animals sensitized by globulin. Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
.	.	1·0	− −	No reaction of animal
.	.	2·0	− −	No reaction of animal
.	.	2·5	− −	No symptoms in one animal; the other died in shock 4 min. after injection
.	.	3·0	−	No reaction
1·75	1·75	3·5	−	1st horn removed: 1st injection given: severe symptoms. 2nd injection 5 min. later. Good recovery
1·5	1·5	4·5	+ −	1st horns removed. Globulin given in three injections at 5 min. intervals. Severe shock in both animals after 1st injection. Good recovery
1·0	.	7·0	− +	1st horns removed. 1st injection given. No reaction. Given 2nd injection 1½ hr. later. No reaction
1·0	.	.	− +	Severe shock and death of both animals about 3 min. after injection

oop of the duodenum, about 4 cm. from the junction with the pylorus, and $3\frac{1}{2}$–4 hr. were allowed to elapse before the second horn was removed. No attempt was made to ensure that the stomach was empty; the experiments were all performed in the morning, and the animals had not been fed since the previous afternoon. Experiments on the absorption of albumin and globulin were carried out concurrently, and as the work progressed it was found not only that the animals could be desensitized by the absorbed protein, but that desensitization was effected at exactly the same level in the two series. No symptoms of anaphylactic shock were observed in any of these animals. In Tables 1 and 2 it is shown that 6·0 mg. of each protein per 100 g. body weight were required to desensitize the animal; below this level a response was still elicited from the second horn. Each sign (+ or −) gives the result of a single experiment; the + sign indicates a response of the second uterine horn to the antigen, and the − sign no response, or desensitization.

TABLE 1. Animals sensitized by globulin. Globulin injected into the duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	mg. globulin per 100 g.	Reaction of 2nd horn
4·0	+	6·0	− − − −
5·0	+ + + + +	7·0	− − −
5·5	+ +	7·5	− − +

TABLE 2. Animals sensitized by albumin. Albumin injected into the duodenum

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
5·0	+ + +	7·5	+ − −
5·5	+ + − +	10·0	− + − −
6·0	+ − − −		

Though the same amount of albumin and globulin per unit of body weight was required to desensitize the second uterine horn in the albumin- and globulin-sensitized animal respectively, it could not be said that the two proteins were being absorbed at the same rate unless the same quantities of protein were necessary in the two animals when the antigen was introduced directly into the portal circulation. It was to be expected that the quantity of protein required to desensitize would be higher when injected into the intestine than when given into the blood; the alteration of some of the protein in the gut was almost inevitable. This problem was first studied in the guinea-pig sensitized by albumin. After removal of the first horn, albumin in normal saline was injected into the large vein which runs along the caecum near to the point where the small intestine enters. Cessation of bleeding from the puncture was usually rapid: the intestines were then returned to the abdominal cavity and the wound was sewed up. The second horn was tested for sensitivity $1\frac{1}{2}$–2 hr. later. The results are given in Table 3 and show that

the first horn was removed, subsequently to be tested for sensitivity; the globulin was then injected into the mesenteric vein. In the first two experiments 2·5 mg. globulin per 100 g. were given in the second injection: surprisingly, severe reaction occurred in one animal; in neither was the second horn desensitized when the animals were killed 1½ hr. later. In three succeeding experiments, in which in the preliminary injection into the gut 5·0 mg. globulin per 100 g. were also given, 6·0 mg./100 g. were injected into the mesenteric vein. In two animals severe shock and death occurred 4–5 min. after the injection into the blood, and in neither was the uterus desensitized: the first uterine horn of the third animal was found to be insensitive, and in this experiment no symptoms of shock were observed on injection of the protein; this shows that a large dose of globulin into the mesenteric vein is toxic only to an animal appropriately sensitized, a conclusion subsequently borne out by the results of experiments in which even larger amounts of globulin were injected into the portal blood of animals highly sensitized to albumin: in these, as in the non-sensitive guinea-pig injected with globulin, no reaction occurred.

A consideration of the results at this stage led inevitably to the conclusion that the liver of the globulin-sensitized animal must possess a very great capacity to absorb globulin presented to it in the portal blood, and the experiments were continued in which globulin was injected into the animal only by the mesenteric vein, in the certainty that, if the extreme sensitivity of the animal could be overcome by graded injections, ultimately the protein must pass the liver and desensitize the uterus if the total dose was high enough. Since it was known that a small dose of globulin given by the portal route was quite unable to desensitize the uterus, it was thought proper to make the first injection before removing the first horn, and this procedure much facilitated desensitization without shock. The first small amount of globulin was acting while the first horn was being removed; when this had been done, a second injection was given and these two injections could be made while the intestines were exposed for little longer than was required for the ordinary operation of removing the first uterine horn. The wound was then sewed up and the animal was again anaesthetized 1½–2 hr. later, when the third and final injection was given. No reaction was observed after any injection, and desensitization was effected by the very large dose of 20 mg./100 g. The results are given in Table 5.

The fact that at least three times the amount of globulin required by the intestinal route to desensitize the uterus must be given when the protein is injected into the portal blood may be explained either by supposing that globulin in the intestine is absorbed not as globulin, but as a protein which is reduced in molecular size yet is antigenically indistinguishable from globulin, or that globulin is absorbed from the intestine unchanged, but after passing

and show that no desensitization of the second horn was effected by the maximum dose then reached, 7·0 mg./100 g.

The remarkable finding that a globulin-sensitized animal was not desensitized by an injection into the mesenteric vein of an amount of globulin which would desensitize if given by the intestinal route, coupled with the tendency of these animals to die in severe shock after the intravenous injection, suggested that the paradox was not a true one, and was due to the comparatively crude attempts at desensitization when the protein was injected into the blood. It will be recalled that desensitization of the globulin-sensitized animal by globulin injected into the intestine was effected with no symptoms of shock; this was due presumably to the steady slow absorption of the protein, quite different from the sudden influx of the most carefully graded injections. It was argued that the effect of injected antigen on some sensitive cells in the wall of the liver vessels might cause severe spasm of these vessels and so slow down the blood flow through the liver as to allow time for the injected globulin to be absorbed and inactivated, if not digested, by cells in the liver. It is well known that the liver can metabolize amino acids, and it was possible that it might be highly active towards whole protein and change it in such a way that, if sufficient time were allowed, the antigenic power of the protein would be abolished. It will be shown subsequently that there is such absorption and inactivation by the liver of albumin injected into the portal circulation. That the liver must play a more important part than has yet been realized, in the events which follow the injection of the reacting dose of protein into the sensitized guinea-pig, is clear from the death of some of these animals which resulted from the injection of globulin into the mesenteric vein, without desensitization of the uterus: presumably the antigen was held in the liver and some reaction took place there of sufficient severity to kill the animal.

The question whether the failure to obtain desensitization of the uterus in the globulin-sensitized animal by a dose of globulin into the mesenteric vein greater than that required by the intestinal route was due to spasm of the liver vessels, was tested in the following manner. Into the duodenum was injected an amount of globulin which was known to be just insufficient to desensitize the animal (5·0 mg./100 g.). It was thought that after some hours the protein would have passed through the liver in the portal blood, and, though the amount was insufficient to desensitize the whole animal, the liver would have received all the protein, presented in minute amounts spread over a long period, and any sensitive cells there would have been completely desensitized: additional globulin, injected into the mesenteric vein, would then go quantitatively past the cells of the liver which were completely 'saturated' and desensitize the uterus and the whole animal. In these experiments 3½ hr. were allowed for the absorption of the globulin from the intestine; at the end of this time the animal was anaesthetized, the abdomen was reopened, and

for albumin. In these experiments the animal was anaesthetized and the first uterine horn was removed: the abdominal wound was then closed and an incision was made in the neck; protein was then injected into the jugular vein. In the two series of animals increasing amounts of protein were given until the level of desensitization was established. Each animal sensitized to albumin received a single injection of albumin into the jugular vein, and, as in the case of the animals given the protein into the mesenteric vein, no symptoms of shock were observed even when sufficient albumin was given to desensitize the animal. Interaction between albumin and the sensitive cells of the albumin-sensitized animal takes place without shock. Reactions of varying severity, in two cases causing death, occurred when the animals sensitized to globulin received this protein into the jugular vein. Globulin is therefore toxic to animals sensitized by it, whether the globulin is injected into the systemic or portal circulations. Globulin injected into the jugular vein, however, was much more effective in desensitizing the plain muscle of the globulin-sensitive uterus than globulin injected by the portal vein. The results are given in Tables 6 and 7 and show that the amounts of protein required to desensitize the albumin- and globulin-sensitized animals are very similar when given into the systemic circulation: if reference is made to Table 3 it will be seen that there is a large absorption of albumin by the liver of the albumin-sensitized animal, twice as much protein having to be given by the mesenteric as compared with the jugular route to desensitize the uterus.

TABLE 6. Animals sensitized by albumin. Albumin injected into the jugular vein

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
0·25	+ +	2·0	+ +
0·5	+ + +	2·5	− −
0·75	+ + +	3·0	− −
1·5	+ +		

TABLE 7. Animals sensitized by globulin. Globulin injected into the jugular vein

mg. globulin per 100 g. 1st	Total	Reaction of 2nd horn	Remarks
			All injections under ether. 1½–2 hr. between injections. Animals killed 1½–2 hr. after (final) injection
	0·5	+ +	One animal died in shock 3 min. after injection
0·25	0·75	+ + +	Two animals showed moderate reaction after 1st injection
	1·5	+	No reaction
0·25	1·5	−	Moderate reaction after 1st injection
0·5	1·5	− −	No reaction
	2·0	−	Moderate reaction
0·33	2·0	−	Moderate reaction after 1st injection
	2·5	− − −	Moderate reaction in two animals: third died in shock 3 min. after injection
0·5	2·5	−	Severe reaction after 1st injection, moderate shock after 2nd

The very large absorption of globulin by the liver of the animal sensitized to this protein was apparently due to the richness of the liver tissues of such

TABLE 5. Animals sensitized by globulin. Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
0·5	1·0	10 0	+ + +	1st injection given, then 1st horn removed. 2nd injection given. Final injection after 2 hr. Killed 2 hr. later. No symptoms of shock
0·5	1·0	15·0	+ + −	As above, but volume of fluid used in final injections was 1·3–2·0 c.c.
0·5	1·0	20·0	− − −	2·0 c.c. given in final injections

the mucous membrane enters not the blood but the lymphatic system, thus by-passing the liver. Against the first view is the fact that the antigenic characteristics of proteins are quickly destroyed in the early stages of breakdown. It seems, moreover, doubtful whether such consistent results would have been obtained in the experiments in which animals were desensitized by albumin and globulin injected into the intestine, if absorption were dependent on enzymic breakdown. The crucial test of the second hypothesis would be injection of globulin into the intestine after ligature of the thoracic duct, not an easy operation to perform in the guinea-pig. In support of the second explanation, however, is the remarkable failure to desensitize the liver cells in those experiments in which globulin was introduced into the intestine in amount nearly sufficient to desensitize the whole animal: if the globulin from the intestine entered the blood by way of the lymphatics and the thoracic duct, then the liver cells would not be preferentially desensitized; and when an injection of globulin was given into the portal blood the liver cells might still be capable of such a profound reaction as actually occurred in three animals out of the four tested. The explanation of the death of the animals, in these and other experiments in which the uterus was not desensitized after enough protein had been given by the portal route to kill them, may be found in the work of Dale, Dragstedt and others (for a review see Dragstedt [1941]), in which it was shown that a toxic substance, almost certainly histamine, was liberated from tissues in sensitized dogs and guinea-pigs following the reacting dose of antigen. In our experiments recorded above in which death occurred, presumably histamine was liberated from cells in the liver. Even though the globulin was not in sufficient amount to pass this organ and desensitize the uterus, the liberated histamine might enter the systemic circulation in sufficient quantity to cause death with symptoms very closely resembling those due to generalized anaphylactic shock.

The absorption of albumin and globulin by the liver

At this stage it seemed advisable to assess the capacity of the liver of the globulin-sensitized animal to absorb globulin, and also to determine whether any special affinity was shown by the liver of the albumin-sensitized guinea-pig

guinea-pig which had been sensitized by globulin. it was of interest to determine whether the amount required by the intestinal route was also smaller. The results of the administration of globulin to serum-sensitized animals by both routes are given in Tables 8 and 9 and show that the amount of globulin which must be injected into the intestine of the serum animal to desensitize it is less than one-tenth of that needed by the globulin-sensitized animal. As might be expected when such small amounts of protein were being used in a biological test of this nature, the results were not sufficiently clear to afford evidence in the serum-sensitized animal as to whether globulin after absorption enters the blood capillaries or the lymphatics. and it is doubtful how much significance must be attached to the two experiments in which desensitization was effected by 0·05 mg. protein per 100 g. given into the duodenum. a level at which desensitization was not effected when the globulin was injected into the portal blood.

SUMMARY OF PART 1

1. The absorption of horse serum and of albumin and globulin from the intestine of sensitized guinea-pigs has been investigated. The quantity required to desensitize an animal when the protein was injected into the intestine was compared with that needed when the protein was introduced directly into the portal blood of another animal. The reaction of the isolated uterus was the criterion of desensitization.

2. It is shown that the three proteins, or substances which are antigenically indistinguishable from them. may be absorbed from the intestine.

3. Animals sensitized by albumin require a greater quantity of albumin to be introduced into the intestine to effect desensitization than is needed when the protein is injected into the portal blood. The reverse was found when the desensitization of the globulin-sensitized animal was effected by globulin.

4. The larger quantity of globulin necessary to desensitize the globulin animal when given into the portal blood. compared with that necessary by the intestinal route. has been attributed to fixation of globulin by the sensitized liver.

5. Albumin must be injected into the portal blood of an albumin-sensitized animal in approximately twice the amount required with injection into the jugular vein, to desensitize the uterus; but it is shown that fixation of albumin by the liver of such an animal is much less than that of globulin by the liver of the globulin-sensitized guinea-pig.

6. Fixation of globulin by the livers of sensitized animals appears to be determined by the type of antibody present in the cells.

an animal in globulin antibody, and not to some special characteristic of globulin itself. This was shown by injecting globulin by the portal route into animals which had been sensitized by an intraperitoneal injection of 0·1 c.c. of whole horse serum 2–3 weeks previously. This series was started by giving, in graded doses, amounts of globulin approaching those found necessary to desensitize the globulin-sensitized animal. All the early results showed de-sensitization of the uterus, and the amount was gradually reduced until it was found, not only that globulin passed through the liver of the serum-sensitized animal with remarkable ease, but that the total quantity required to desensitize such an animal was less than one-thirtieth of that needed by the globulin-sensitized guinea-pig when the protein was given by the portal route, and about a third of that required by the globulin-sensitized animal when given by the jugular vein. Desensitization of the serum-sensitized animal was effected by such small amounts of globulin given by the portal route, that it did not seem worth while to perform any experiments in which the protein was given by the jugular route: if there is any absorption of globulin by the liver of the serum-sensitized animal it probably lies within the limits of experimental error of the method. Another fact of interest which emerged as these experiments proceeded was that desensitization must have been effected by the small initial amount of protein used when a large quantity was given by graded doses, yet no symptoms were observed.

Since the serum-sensitized animal required so small an amount of globulin given by the portal route to saturate the receptive cells as compared with the

TABLE 8. Animals sensitized by serum (2–3 weeks). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		No symptoms of shock in any animal. Animals killed 1½–2 hr. after (final) injection
0·5	1·0	12·5	–	1st injection of globulin given. 1st horn removed. 2nd injection given. Final injection 1½–2 hr. later
0·5	1·0	10·0	– –	Injections as above
0·5	1·0	7·5	– –	Injections as above
0·5	1·0	4·0	– –	Injections as above
0·5	.	1·5	–	1st horn removed. 1st injection given; 2nd injection 2 hr. later
.	.	0·5	– – – +	1st horn removed. Globulin injected
.	.	0·25	+ – –	1st horn removed. Globulin injected
.	.	0·1	+ + –	1st horn removed. Globulin injected
.	.	0·05	+ + ⊥	1st horn removed. Globulin injected

TABLE 9. Animals sensitized by serum (2–3 weeks). Globulin injected into the duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	Remarks
		Animals killed 3½–4 hr. after injection
1·5	– – – +	
0·5	– – – +	
0·25	+ + +	
0·1	+ + –	
0·05	+ – – +	

,nimals caused the death in shock of some, while others receiving the same dose were unaffected, though the first uterine horns appeared equally sensitive. The same thing was noticed in other series which will be described later and referred to in the discussion. Except when injecting the smallest quantities of serum into the portal blood of the globulin-sensitized animal, the protein was given usually in two doses, the first after removing the first horn, the second 1½–2 hr. later; the animals were killed 1½–2 hr. after the second injection. The amount of serum required to desensitize was found to be so small that in order to get below the minimum dose, rather large animals were used and these were given 0·01 c.c. of serum, the smallest quantity which could be measured with any degree of accuracy; from the nature of the problem under investigation any use of diluted serum was prohibited. This accounts for the somewhat curious doses per 100 g. body weight given at the minimum levels. The results are given in Table 10, and it will be noted that death in shock occurred from even 0·01 c.c. of serum, also that 0·0075 c.c. of serum per 100 g. is as effective in desensitizing as 0·1 c.c., or fourteen times as much: at no point above the minimum, up to the maximum dose given, was serum always

TABLE 10. Animals sensitized by globulin. Serum injected into the portal blood

c.c. serum per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
			1st injection given after removal of 1st horn. 1½–2 hr. between injections. Animals killed 1½–2 hr. after (final) injection
0·01	0·10	– – –	1st injection caused severe reaction in one animal, moderate in others. No symptoms after 2nd injection
0·01	0·075	– – – –	No reaction in any animal
0·0075	0·06	–	No reaction in any animal
.	0·05	– –	No reaction in any animal
.	0·05	– –	Death 3 min. after injection
0·0075	0·032	–	Death 3 min. after 2nd injection
0·0075	0·02	– –	Moderate reaction in one animal after 2nd injection
.	0·0075	– – –	No reaction
.	0·0029	–	The last five animals received 0·01 c.c. serum. The last animal
. .	0·0023	± –	died in shock 3 min. after injection
.	0·0020	–	
.	0·0022	∓	

TABLE 11. Animals sensitized by serum (2–3 weeks). Serum injected into the portal blood

c.c. serum per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
			1st injection given after removal of 1st horn. 1½–2 hr. between injections. Animals killed 1½–2 hr. after (final) injection
0·01	0·05	– ± – –	Severe reaction in one animal after 1st injection
0·01	0·035	– – –	No reaction in any animal
0·005	0·02	– – –	No reaction in any animal
.	0·015	–	Death 5 min. after injection
.	0·0075	.	Shock and death in 5 min. 2nd horn not examined
.	0·0075	– – –	No reaction
.	0·0025	–	The last three animals received 0·01 c.c. serum. No reaction
.	0·0023	–	
	0·0018	–	

1. Serum as a desensitizing antigen

Nearly forty years ago Hardy [1905] performed experiments which threw doubt upon the dual nature of the protein of blood serum. Having shown that there was no movement of protein in serum when a current was passed through it, Hardy concluded that alkali-globulin could not be present in serum: further, serum could pass through the pores of a clay cell which retained globulin. Hardy was therefore of the opinion that globulin is not present in untreated serum, and suggested that some complex protein breaks up to yield globulin when serum is diluted or precipitated by salts. Since then, so much work has been carried out on the albumin and globulin of blood serum, particularly in relation to their still further subdivision, that the significance of Hardy's original work has been rather lost to sight. A difficulty which was apparent until recently, the implication that in the break-up of serum protein to yield globulin a larger molecule results, has been met by the work of Svedberg & Sjögren [1930], in which it was shown that when globulin breaks up into euglobulin and pseudoglobulin very large particles are formed. Macfarlane [1935], as a result of ultracentrifuge experiments, concluded that serum is unhomogeneous as regards particle size, and while globulin may be present it is not possible to regard the lighter molecular fraction as identical with isolated albumin. The question seems to be incapable of direct proof if any chemical process and even dilution breaks up the supposed serum protein, but it was thought that an investigation into some of the antigenic properties of blood serum might throw light on the problem. The remarkable affinity for globulin of cells in the liver of the globulin-sensitized animal gave a means of approach, by determining the amount of serum required to desensitize when given by the portal route and comparing this with the globulin content of serum as found by analysis. When the literature on the latter point was consulted, wide discrepancy was found in the figures for the albumin and globulin content of horse serum, and though the horse is usually considered to be one of those animals in which the globulin exceeds the albumin in amount, not all authors were in agreement upon this. In work of the nature of that to be undertaken it was desirable to discount small differences and take outside figures: it was assumed therefore that 8 g./100 c.c. would represent the greatest amount of total protein which would be present in any sample of horse serum, and that the albumin/globulin ratio does not differ significantly from unity, i.e. 0·1 c.c. of serum contains not more than 4 mg. of albumin and 4 mg. of globulin.

Desensitization by serum of animals sensitized to globulin

As in the case of globulin-sensitized animals receiving globulin into the mesenteric vein the injection of serum by the same route in similarly sensitized

in an animal after the first injection, the initial dose was reduced and desensitization was then effected without difficulty in a number of experiments until a particularly sensitive animal made further reduction advisable. It will be seen from Table 14 that the second uterine horn was regularly desensitized until the level of globulin was below the minimum found necessary when globulin alone was injected into the *jugular* vein, and even at the low level of 0·5 mg./100 g. only two animals out of five were still sensitive. It would seem that the presence of albumin not only allows globulin to pass freely through the liver of the globulin-sensitized animal, but actually reduces somewhat the amount of globulin required to desensitize a given weight of tissue. If the border-line of desensitization by the two proteins combined is taken t 0·5 mg. of each protein per 100 g., a level which is certainly not too low, nd the usual allowance is made for the salt content of the protein, there is lose agreement with the albumin and globulin content as found by analysis of serum at its minimum desensitizing level in the globulin-sensitized animal.

TABLE 14. Animals sensitized by globulin. Mixture of albumin and globulin injected into the portal blood

mg. albumin and globulin per 100 g.				
1st	2nd	Total	Reaction of 2nd horn	Remarks
				1st injection given. 1st horn removed. 2nd injection given. Final injection after 2 hr. Animals killed 1½–2 hr. after final injection
0·5	1·0	4·5	– –	Severe reaction in one animal after 1st injection
0·25	0·75	4·0	~	No reaction
0·25	.	.	.	Shock and death in 3 min. 2nd horn not examined
0·15	0·6	4·0	– –	No reaction
.		3·0	~	Severe reaction, but good recovery
0·15	0·6	3·0	· –	No reaction
0·15	0·6	2·0	–	No reaction
0·05	0·1	2·0	– –	No reaction
0·15	.	1·0	–	Severe reaction. Good recovery. 2nd injection 2 hr. later
0·05	0·1	1·0	– –	No reaction
0·05	0·1	0·5	– – – –	No reaction

Desensitization by serum of the animal sensitized to albumin

The efficacy of serum as a desensitizing agent when introduced into the portal blood was investigated in a series of animals sensitized by albumin, with a view to discovering whether this desensitization might be accounted for solely on the known albumin content of serum. It was anticipated that the discrepancy would be even greater than in the case of the globulin animal desensitized by serum, since, unlike the latter animal whose isolated uterus is not responsive to albumin *in vitro*, there is often, but not invariably, some reaction when 5 mg. of globulin are placed in the bath containing the isolated uterus sensitized to albumin. Though no cross-reaction was found *in vivo* when the albumin-sensitized animal was injected with globulin (Table 13), the experiments made *in vitro* suggested that there would be a greater tendency in

effective. Where failure to desensitize occurred, there was no doubt on the matter, a large response was obtained when the antigen was added to the bath. The same phenomenon was observed in the corresponding series where serum-sensitized animals received injections of serum into the mesenteric vein. No explanation can be offered as to why serum is haphazard in its action as a desensitizing antigen.

If the border-line dose of globulin required to desensitize a globulin animal when given by the portal route is taken at 15 mg./100 g., and it is possibly even higher, and allowance is made for the 15% of ammonium sulphate in the globulin used, and the figure is then compared with the globulin content of the amount of serum used for desensitization at the minimal point (0·0075 c.c./100 g.), the ratio is rather greater than 40 to 1. Doubt was at once thrown on the presence of globulin in serum, but it was possible that albumin in serum might be a desensitizing agent in the globulin animal. Though experiments had shown that uterine horns of globulin-sensitized animals showed no response when 10 mg. of albumin were placed in the bath it was possible that there might be some action *in vivo*. Animals sensitized to globulin were therefore injected with albumin through the mesenteric vein, and others received the protein into the duodenum. The converse experiments were performed about this time and may conveniently be taken here; albumin-sensitized animals were given globulin by the portal and intestinal routes. As is shown in Tables 12 and 13, there were no cross-reactions. Though it was now

TABLE 12. Animals sensitized by globulin. Albumin injected into the portal blood and duodenum

mg. albumin per 100 g.	Reaction of 2nd horn	Remarks
6·0	+ + +	Injection into portal blood. No reaction. Animals killed 2–3 hr. later
6·0	+ + +	Injection into duodenum. Animals killed 4 hr. later

TABLE 13. Animals sensitized by albumin. Globulin injected into the portal blood and duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	Remarks
8·0	+ + + +	Injection into portal blood No reaction
6·0	+ +	Injection into duodenum
15·0	+ +	Injection into duodenum. Volume of injection fluid was 2·0 c.c.

clear that neither globulin alone, nor albumin alone, could account for the desensitizing power of serum for the globulin animal, the possibility remained that these two proteins in conjunction might effect what neither could carry out singly. Animals sensitized to globulin were therefore injected with a mixture of albumin and globulin ('synthetic serum') by the portal route. The proteins were mixed each day and dissolved in normal saline, the usual concentration being 10 mg. of each protein per c.c. Graded injections were used, the first being given before, the second after removal of the first horn, and the third about 1½ hr. later. When death or severe symptoms occurred

protein was given into the portal blood. The series already recorded (Table 3) was adequate for the purpose when the experiments were performed, but, in order to compare the action of synthetic serum with albumin alone in the albumin-sensitized animal, a greater number of results was desirable at the border-line. The extension of these experiments is shown in Table 16, and it will be seen that there is no material difference, the border-line still standing at 4·5 mg./100 g.

TABLE 16. Animals sensitized by albumin. Albumin injected into the portal blood

mg. albumin per 100 g.	Reaction of 2nd horn
3·0	÷ + ÷ +
4·0	+ ÷ ÷ −
5·0	− − − −

TABLE 17. Animals sensitized by albumin. Synthetic serum injected into the portal blood

mg. albumin and globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
				Method of injection as in Table 15
0·1	0·62	4·0	− − −	No reaction
.	.	3·0	−	Death in shock 3 min. after injection
.	.	3·0	−	Severe reaction, but good recovery
0·15	0·62	3·0	− − −	No reaction
0·15	.	3·0	⊥	Severe reaction after 1st, none after 2nd injection
0·1	.	2·0	− ÷	No reaction

The experiments with synthetic serum (Table 17) show that the border-line is 3·0 mg. of each protein per 100 g., sufficiently below that with albumin alone to indicate that the globulin did perceptibly augment the desensitizing action of the albumin. The influence of the globulin, however, was much more definite in the production of anaphylactic shock, to which the albumin animals were subject when injected with the synthetic serum, but not at all when injected with globulin or albumin alone. Calculation shows that, in the albumin animal, natural serum is also perceptibly more effective in desensitization than can be accounted for by its content of albumin and globulin; but the difference is small. Altogether, the effect of globulin, in enhancing the desensitizing action of albumin by the portal blood on the albumin-sensitive animal, is far less than that of albumin in enhancing the desensitizing effect of globulin by the same route on the globulin-sensitive animal. To determine whether the globulin in synthetic serum merely abolishes the capacity of the liver cells to absorb albumin, five experiments were performed in which synthetic serum in graded doses was injected into the jugular vein of albumin-sensitized animals, at the level of 2·0 mg./100 g. In two of these the second horn was still sensitive, and comparison with Table 6 will show that in the albumin animal the presence of globulin does little to reduce the absolute amount of albumin necessary to desensitize the tissues.

this direction than when the converse experiments with globulin-sensitized animals were performed. Such was not discovered: there was found to be a greater desensitizing action on the albumin animal by serum and by a mixture of albumin and globulin than could be accounted for on the albumin content; but the influence of the globulin was much less marked, quantitatively, than was that of the albumin in the converse experiments. The results in this series are given in Table 15. Qualitatively, the action of serum, or of a mixture

TABLE 15. Animals sensitized by albumin. Serum injected into the portal blood

c.c. serum per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
	–			When two injections given, 1½–2 hr. interval. When three injections, 1st and 2nd given immediately before and after removal of 1st horn. Animals killed 1½–2 hr. after final injection
	..	0·075	–	No reaction
0·015	.	0·05	– – –	No reaction
0·0075	0·015	0·05	– –	No reaction
0·015	.	0·042	+	No reaction
0·015	.	0·042	+	Death in shock 4 min. after 2nd injection
0·015	.	.	. .	Death in 3 min. 2nd horn not examined
0·0075	0·015	0·042	– +	No reaction
0·0075	.	0·042	–	Severe reaction after 1st injection; good recovery. Moderate reaction after 2nd injection. Normal when killed 2 hr. later
0·003	.	0·042	–	Severe reaction after 1st injection; good recovery. Reaction and death 3 min. after 2nd injection
.	.	0·035	+ +	Severe shock in both animals; death in 3 min.
.	.	0·035	+ +	Moderate reaction in one animal
0·0075	0·015	0·035	+	No reaction

of albumin and globulin, on the albumin-sensitized animals was very different from that of albumin alone. While no death occurred, or even any sign of shock, when the albumin animal was desensitized by a single injection of albumin, the use of serum or 'synthetic serum' was found to be dangerous to the animal and desensitization had to be attempted by graded doses. Even with the utmost care and the use of a very small initial dose, death occurred in a number of cases when serum was injected: 'synthetic serum' was less toxic. The border-line dose of serum necessary to desensitize was found to be 0·042 c.c./100 g. Below this level the uteri were still sensitive: in the six experiments above this level there was no failure to desensitize, but more observations would be required to make certain that serum does not show its haphazard action in these animals as it does in those sensitized to serum and to globulin. The amount of albumin necessary to desensitize an albumin animal, when given by the portal route, may be taken to be 3·8 mg./100 g. (from Table 3) after correcting for the ammonium sulphate: 0·042 c.c. serum should contain not more than 1·7 mg. albumin. There is thus a large discrepancy in favour of serum, which might be attributed to the contained globulin. Before starting to test the action of synthetic serum on animals sensitized by albumin, it was important to have more exact information as to the amount of albumin required to desensitize these animals when the

TABLE 20. Animals sensitized by serum. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn	Remarks
1·25	– – –	Animals sensitized 15–20 days
0·95	– – –	Animals sensitized 30–35 days
0·80	– – –	Animals sensitized 15–20 days
0·66	– – – –	Animals sensitized 30–35 days
0·33	± – – –	Animals sensitized 30–35 days
0·16	– – – –	Animals sensitized 15–20 days

corresponding to a globulin content of 34·8 mg., whereas the serum animal (Table 9) was desensitized by 0·5 mg. globulin per 100 g. The very marked difference shown by serum as a desensitizing agent when given by the intestine, as compared with its action when injected into the blood, suggests either that the serum protein is a complex, in which albumin and globulin have no independent existence, and that the cells of the intestinal mucosa will pass this complex protein only with difficulty, or that albumin and globulin, which together make a more efficient desensitizing agent than either alone when injected into the portal blood, impede one another in their passage through the wall of the intestine. Interference might also be caused by the lipins in serum, delaying the passage of albumin and globulin through the intestinal mucosa, though this factor would not account for the great difference between the globulin and serum-sensitized animals, the latter, which required the smaller amount of globulin, needing the larger amount of serum in the intestine. The problem was first investigated in the globulin-sensitized animal. Serum was shaken three times with an equal volume of ether, the ether extracts were combined, measured, and portions were evaporated to dryness in a series of basins which thus contained lipins from a known amount of serum. For use, the lipoid residue was rubbed up with a measured quantity of normal saline which was added to that in which the albumin and globulin were dissolved. The mixture was then injected into the duodenum after removal of the first uterine horn, and the animals were killed 4 hr. later. An amount of globulin was chosen which was more than sufficient to desensitize the globulin animal, and the same weight of albumin was also present: the mixture contained lipins from approximately 0·3 c.c. serum per 100 g., that amount of serum which was the border-line dose for desensitization. From Table 21 it will be seen that desensitization was not effected, nor was it

TABLE 21. Animals sensitized by globulin. Mixture of albumin and globulin, with and without lipins, injected into the duodenum

mg. albumin and globulin per 100 g.	Reaction of 2nd horn	Remarks
7·5	– – – –	Protein and lipins
7·5	– – – –	No lipins

effected when the albumin and globulin were injected without lipins; the latter can have contributed nothing to the result. Albumin seems to have an inter-

Desensitization by serum in the intestine

Serum having been shown, when injected into the portal blood, to de
sensitize albumin and globulin animals at levels which could not be accounted
for by its content of albumin and globulin respectively, it seemed advisable
to determine whether a similar phenomenon occurred when serum was in
jected into the intestine, i.e. does serum desensitize an albumin animal from
the intestine by virtue of the albumin content as given by analysis, and
similarly with globulin for the globulin animal, using for comparison the
figures given in Tables 2 and 1? After removal of the first uterine horn, serum
was injected into the duodenum of these animals, which were killed about
4 hr. later. When a sufficiently large amount of serum had been given, de
sensitization was effected, always without any symptoms of shock. Injection
of serum into the blood of both albumin and globulin animals may be followed
by severe shock and death, but evidently during the slow absorption of serum
from the intestine desensitization is sufficiently gradual to obviate any
symptoms. The results are given in Tables 18 and 19. In the case of the albumin

TABLE 18. Animals sensitized by albumin. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn
0·66	− + − −
0·5	− − + +
0·33	− + − +
0·16	+ + + +

TABLE 19. Animals sensitized by globulin. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn
0·66	− + − −
0·33	+ − − +
0·16	+ − + +

animal it will be seen that the border-line dose of serum is about 0·5 c.c./100 g.
corresponding to 20 mg. of albumin. In Table 2 the minimum desensitizing
dose of albumin by the duodenum is 6·0 mg., less than one-third. This shows
that, while serum injected into the blood is a more efficient desensitizing agent
than can be accounted for by its albumin content, the position is reversed
with injection into the intestine. A similar result was obtained when serum
and globulin were injected into the intestine of the globulin animal, though
the difference was less marked. Here the border-line dose of serum is about
0·33 c.c./100 g., corresponding to a globulin content of 13·2 mg., while globulin
alone by the intestine desensitizes at 6·0 mg./100 g. The desensitization of the
serum-sensitized animal by serum in the intestine was also studied: again,
desensitization was effected without symptoms of shock, but the amount
required was much greater than in the albumin and globulin animals, and
the discrepancy as regards the globulin was enormous. The results given in
Table 20 show that the border-line dose of serum is about 0·87 c.c./100 g.,

from the time of injection, i.e. at a time when the albumin, if acting independently, could not cause any response to this protein in the isolated uterus [Dale & Hartley, 1916]. Seven animals were given an injection of 2·5 mg. albumin and 2·5 mg. globulin in 0·2 c.c. normal saline and were examined 12–19 days later. The first uterine horn of each animal was removed and 0·5 mg. globulin per 100 g. was then injected into the mesenteric vein. One animal died in shock about 4 min. later. The others showed no symptoms, and received a further injection of globulin 1½ hr. later, 1·0 mg./100 g. A total quantity of 1·5 mg. globulin per 100 g. was chosen for these and similar experiments to be described later, since it was felt that any failure of this amount to desensitize the animals would be significant in differentiating the globulin from the serum type of animal (see Tables 4 and 8). No symptoms were observed, and the animals were killed about 1½ hr. later. The second horns were in every case insensitive to 0·1 c.c. serum placed in the bath. The first horns had shown no response to 5 mg. albumin, but after changing the Ringer's solution gave large responses to 1 mg. of globulin. The uteri of the animals sensitized by serum and by synthetic serum are therefore, at this stage of sensitization, qualitatively identical; and the desensitization action on the two groups of globulin introduced through the portal circulation seemed also to be the same, with the exception that in the group sensitized by 'synthetic serum' one died in anaphylactic shock, while none of those sensitized with natural serum has ever shown any symptoms following an injection of globulin. The conclusion seems inescapable that albumin and globulin given at the same time act in conjunction as sensitizing antigens to give rise to a sensitized animal apparently identical with one which has received serum, up to 19 days from the time of injection: the albumin in the animal receiving synthetic serum could not be shown to have influenced the isolated uterus, but it had influenced certain receptive cells, presumably in the liver, so as to allow a small amount of globulin to pass and to desensitize the uterus.

Another series of animals was sensitized by albumin and globulin, 2·5 mg. of each protein, but the animals were kept for a longer period and were examined between the 30th and 35th days. The first uterine horns were indistinguishable from those of the previous series; there was no reaction to albumin, a large response to globulin. Differences, however, appeared in the reactions of some of the animals as a whole, as may be seen from Table 23. Two animals died in shock as a result of the second injection, even though a small amount of globulin had been injected 1½ hr. previously. Of the eleven animals which survived and which were killed 1½–2 hr. after the final injection, four were not desensitized. This suggests that the influence of the albumin given in the sensitizing injection had waned, and that these four animals were becoming like those sensitized to globulin alone. In the remaining seven animals the influence of the albumin had persisted, and these were indis-

fering action on the passage of globulin through the intestine of the globulin-sensitized animal. This result became all the more difficult to explain whe it was found that a similar effect was not observed in experiments on th albumin and serum-sensitized animals. Lipins were not added, since the resul of the experiments on the globulin-sensitized animals could not be attribute to them. There was no interference when albumin and globulin togethe were absorbed from the intestine of the albumin and serum animals. Seru seems to behave as a unique desensitizing agent when operating from th intestine.

TABLE 22. Animals sensitized by albumin or serum. Mixture of albumin and globulin injected into the duodenum

mg. albumin and globulin per 100 g.	Reaction of 2nd horn	Remarks
7·5	− − + −	Animals sensitized by albumin
7·5	+ − − −	Serum-sensitized animals (15–20 days)

2. Serum as a sensitizing antigen

Though the experiments described in the preceding section threw doubt o the separate existence of albumin and globulin in blood serum, none we conclusive. It remained to study the antigenic properties of serum from th opposite direction, and determine how far these might agree with thos exhibited by albumin and globulin together, when acting as sensitizin antigens. When an animal which has been given an injection of serum fir becomes sensitive, the isolated uterus is responsive to globulin, not to albumin this was shown by Dale & Hartley [1916], and was confirmed on the fir uterine horns of the animals used in Table 8. No reaction to 5 mg. albumi was observed, but if 1 mg. of globulin was placed in the bath a large, usuall maximal, response was obtained. The uteri of these animals were thus indif tinguishable, qualitatively, from those of animals sensitized by globulin. Whe the animals thus sensitized by serum, at a stage at which only sensitivenes to globulin was present, received an injection of globulin into the portal bloo the animals as a whole were very different from globulin-sensitized animal since they were readily desensitized, without symptoms, by a small dose o globulin. The difference in the behaviour of the intact animals sensitized b globulin and by serum respectively, the antibodies in whose uteri were re sponsive only to globulin (or to serum, which causes a reaction in all type but does not differentiate them), could only be attributable to the presence o albumin in the serum, either free or combined with globulin to form serum protein. Experiments were therefore performed to determine whether animals sensitized by a mixture of albumin and globulin behaved like serum-sensitized animals, both as regards the reactions of their isolated uteri and the de sensitizing effect on them of injected globulin, when examined under 21 days

both as regards the reactions of the isolated uterus, and the response of the whole animal to injected globulin, from the 15th to the 35th day, thus differing sharply from the animal sensitized by a mixture of albumin and globulin which is 'unstable'. Experiments were undertaken to determine whether the serum-sensitized animal is stable over a longer period. A series of animals received an intraperitoneal injection of 0·1 c.c. serum and was examined between the 49th and 51st days. Eight animals were used: in no case was there any response of the isolated uterus to albumin, but the usual large reaction followed the introduction of globulin to the bath. Four of the animals received an injection of globulin, 0·15 mg./100 g., after removal of the first horn, and in one a severe reaction followed, but the animal recovered and was normal 1½ hr. later, when this animal, with the others, received the second injection of globulin to bring the total to the standard figure of 1·5 mg./100 g. When the animals were killed 1½–2 hr. later the uterine horns were all sensitive to globulin. The other four animals received the usual preliminary injection of 0·15 mg. globulin per 100 g. and no shock was observed. In the second injection a larger amount of globulin was given to bring the total to 5 mg./ 100 g.; one animal suffered from severe shock after the second injection but made a good recovery. When the animals were killed 1½–2 hr. later, all were found to be desensitized. The serum-sensitized animal is thus not stable indefinitely, but requires, with increase of time from the date of sensitization, an increased amount of globulin to discharge the sensitivity.

Sensitization by diluted serum

The differences observed between animals sensitized 30–35 days by serum and by synthetic serum might have been due to the quantities of albumin and globulin in the latter being present in a different ratio from that in which they exist in serum. It would not necessarily imply, therefore, that albumin and globulin are present in serum in some type of combination differing from one which might be formed when albumin and globulin are remixed after separation. Sensitization of animals by diluted serum would throw light on this question, since, if serum contains a complex protein, the albumin and globulin which can be isolated from it must be dissociated at some stage by simple dilution of the serum, or by the addition of a salt such as ammonium sulphate, or, possibly, by the operation of both factors. Serum diluted 1/10 with water and with normal saline was used. The dilutions were made in the morning and the serum was allowed to stand until the afternoon at room temperature. After stirring, 1·0 c.c. was injected into each guinea-pig and the animals were examined between the 30th and 35th days. The reactions of the first horns *in vitro* were the same as those of all the other 30–35 day serum or synthetic-serum animals; there was no response to albumin, a large response to globulin. After removal of the first horns 0·15 mg. globulin per 100 g. was injected into

TABLE 23. Animals sensitized 30–35 days by a mixture of albumin and globulin.
Globulin injected into the portal blood

mg. globulin per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		1st injection after removal of 1st horn. 2nd injection (when given) 1½–2 hr. later. Animals killed 2 hr. after final injection
.	1·5	+	Severe shock and death in 4 min.
.	1·5	–	No reaction
0·15	1·5	+ +	Death in shock 3–4 min. after 2nd injection
0·15	1·5	– – + – + – + + – –	No reaction

tinguishable from those animals examined 12–19 days after sensitization with synthetic serum.

It remained to examine a number of animals sensitized by natural serum 30 days and over, to determine whether there was any difference between them and the 15–20 day serum animals as regards the reactions of the isolated uteri and of the animals as a whole. Twelve such animals were used. After removal of the first horn an injection of globulin was given into the mesenteric vein, 0·15 mg./100 g.; 1½ hr. later the second injection was given, bringing the total to 1·5 mg./100 g. The animals were killed 1½–2 hr. later. All the animals survived, with no sign of shock after the first or second injections: the uteri of all were desensitized. As regards the whole animal therefore, there is no difference between this series and those sensitized only 15–20 days by serum. Nor could any difference be detected in the first uterine horns of the 30–35 day serum-sensitized animals; they did not react to albumin placed in the bath, but gave large responses to globulin. This finding conflicts with that of Dale & Hartley [1916] who showed that the uterus of the serum-sensitized animal 28 days and upwards, unlike the 15–20 day animal, is responsive *in vitro* to a small amount of albumin: these workers also found that, after a larger quantity of albumin had been added to the bath and the sensitivity of the uterus to this protein had been entirely discharged, the muscle still responded to euglobulin. Though the volume of Ringer's solution used in our experiments was larger, no explanation is possible on the lines of a lower concentration of albumin. The difference is essentially qualitative: Dale & Hartley found the uterus of the 31 day serum-sensitized animal more sensitive to albumin than to euglobulin, and stated: 'There was some indication that concurrently with the late development of sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st day, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction.' In our experiments 1 mg. of albumin caused no response, nor did 5 mg., but after changing the Ringer's solution there was a large, usually maximal response to 1 mg. of globulin. The serum-sensitized animal appears to be 'stable'

mainder before removing the uteri, with a view to aiding desensitization
ithout death in shock: the G-A animals were found, indeed, to be very
usceptible to globulin, and death often occurred. Of the eight first uterine
orns examined, one showed no response to albumin or to globulin; in another
here was a maximal response to 1 mg. of albumin and none to globulin: the
ormer had failed to become sensitized to either protein, the latter had evi-
ently not been sensitized by the globulin, and the cells had been left free to
cquire albumin sensitivity. The remaining six uteri showed no response to
mg. of albumin: 5 mg. were then added with no result: the Ringer's solution
was changed and a large response to 1 mg. of globulin occurred in each case.
The uteri of the eight succeeding animals, removed after the first injection of
globulin, all responded to 1 mg. of globulin, but showed no response to 5 mg.
of albumin. In fourteen animals the uteri were responsive to globulin alone
and therefore were indistinguishable from those of animals which had been
sensitized only by globulin: The results of the globulin injection (Table 25)

TABLE 25. Animals sensitized by albumin 19 days after sensitization by
globulin (G-A animals). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
First uterine horns removed before injection of globulin				
.	.	0·5	—	Severe shock; death in 3 min.
.	.	0·15	—	Severe shock; death in 3 min.
0·15	.	1·5	—	2nd injection 1¼ hr. after 1st. Moderate shock. Animal recovered, then became weak and died ¾ hr. later
0·15	.	1·5	—	Severe shock after 1st injection. Severe shock after 2nd injection 1¼ hr. later; death in 3 min.
0·15	.	1·5	— —	No reaction. 1¼ hr. between injections
First injection of globulin before removal of 1st horn. 2nd injection given immediately after; final injection 1¼–2 hr. later				
0·15	0·35	1·5	— —	No reaction
0·05	0·35	1·5	— — —	No reaction
.	.	0·15	.	Death 3 min. after injection
0·05	0·10	1·5	.	Death 5 min. after final injection
0·05	0·10	1·5	.	Death 15 min. after final injection
				2nd horns of last three animals not examined

show that the animals differed from animals sensitized by an injection of
globulin only, since, of the seven animals which survived the usual 1½–2 hr.
period after the second injection, all but one were desensitized. The injected
albumin therefore had influenced the whole animal; though it was not de-
tectable by the reaction of the first uterine horn, it had affected certain other
sensitized cells, probably in the liver, in such a way that they allowed a
sufficient amount of globulin to pass them without fixation, and so to de-
sensitize the plain muscle elsewhere. The G-A animals were also much more
difficult to desensitize without death in shock than were animals which had
been injected only with globulin. A few experiments were performed to
determine whether the albumin could affect the globulin antibody in the

the portal blood; 1·0 mg. globulin per 100 g. was injected 1½ hr. later. The animals were killed after 1½–2 hr., with the exception of one which died in shock 4 min. after the second injection. One animal showed a severe reaction after the first injection, but made a good recovery. Reaction in only two animals may not be significant by itself to differentiate this series from those animals which were sensitized by undiluted serum, but the second uterine horns of four of the twelve animals were not desensitized. All of the twelve animals sensitized with injections of natural serum given 30–35 days previously were desensitized by such globulin injections, and none showed any symptoms of shock in the process. The different results observed after sensitization with diluted serum, in which all the constituents of the natural serum were present in the sensitizing dose, in the same quantities and proportions, and the only change made was the previous dilution of the dose, suggest that the difference was due to the breaking up of some complex in which the proteins exist in the natural, undiluted serum, so that the resulting products gave rise to sensitized animals similar to those sensitized with 'synthetic serum' and kept for the same period (cf. Table 23).

TABLE 24. Animals sensitized 30–35 days by diluted serum.
Globulin injected into the portal blood

mg. globulin per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
0·15	1·5	−	Severe reaction after 1st injection, but good recovery. Serum diluted with NaCl
0·15	1·5	+	Death in shock 3 min. after 2nd injection. Serum diluted with NaCl
0·15	1·5	+ + − −	No reaction. Serum diluted with NaCl
0·15	1·5	+ − − − + −	No reaction. Serum diluted with water

Sensitization by separated injections of albumin and globulin

The complete suppression of sensitivity to albumin in the isolated uteri of animals sensitized by a mixture of albumin and globulin was so remarkable as to merit further investigation. While the non-appearance of albumin sensitivity in the serum-sensitized animals might have been due to the absence of free albumin from untreated serum, no such explanation was possible in the animals sensitized by 'synthetic serum'. Guinea-pigs were therefore sensitized by an injection of 2 mg. of globulin and, after 19 days, when full sensitivity to globulin would have been established in the cells, 2 mg. of albumin were injected into the animals. A further period of 30–33 days was allowed to elapse and the animals (which for brevity will be referred to as G-A animals) were then examined. The first uterine horns of eight of the sixteen animals were removed before any globulin was injected, and when the reactions of these uteri to albumin and globulin had yielded consistent results it was considered permissible to give a small preliminary injection of globulin to the

TABLE 26. Animals sensitized by globulin 31 days after sensitization by albumin (A-G animals). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		

First uterine horns removed before injection of globulin

0·15	.	1·5	– –	No reaction. 1½–2 hr. between injections. Animals killed 1½ hr. after 2nd injection
0·15	.	1·5	–	Animal died in severe shock 5 min. after 2nd injection
0·15	.	1·5	–	Gradually became weak and died 1 hr. after 2nd injection

First injection of globulin before removal of first uterine horn; 2nd injection immediately after; final injection 1½–2 hr. later

0·15	0·35	1·5	–	Severe shock after 2nd injection. No shock after final injection, but was weak when killed 1½ hr. later
0·05	0·10	1·5	–	No reaction; very weak when killed 1¼ hr. after final injection
0·05	0·10	1·5	–	Died 1½ hr. after final injection
0·05	0·10	1·5	.	Died ½ hr. after final injection; 2nd horn not examined
0·10	0·20	.	.	Severe shock and death 5 min. after 2nd injection. 2nd horn not examined

been desensitized: no response was observed when 1 mg. of albumin was placed in the bath. The sensitivity of the A-G animals to albumin had all been discharged by a small amount of globulin. These facts must indicate that the sensitizing dose of globulin, though it had failed to establish any sensitivity in the uterus which could not be discharged by albumin, had nevertheless influenced the albumin antibody so that it was neutralized by globulin *in vivo*. Since it is difficult to conceive of entirely different actions of globulin *in vitro* and *in vivo*, it is also highly probable that had the first uterine horns been tested by the introduction of globulin into the bath before albumin, a maximal response would have been obtained to the former protein, and that the sensitivity would all have been discharged by globulin. More experiments will be required to be certain on this point, but a consideration of the different series of animals sensitized, viz. by serum, 'synthetic serum', globulin followed by albumin, and albumin followed by globulin, must lead to conviction that in all these animals both albumin and globulin had actively participated in antibody formation; the differences exhibited in the reactions of these animals and of their uteri show that the antibody cannot have been the same in every case. Albumin and globulin must influence one another mutually in antibody formation, but the type of antibody which results is dependent upon the conditions under which the proteins are presented to the animal.

DISCUSSION

The uteri of 797 animals were examined. Of these, 665 had been injected previously with 5 c.c. of milk in the routine test for tubercle carried out in the Department of Bacteriology, for which they were kept 4–5 weeks before being killed. The injection of albumin, globulin, or serum was given to these animals at the appropriate time after the injection of milk, so that the date

G-A animal, at a time when it could not cause an antibody to be fixed in the cells if it were the only protein injected, i.e. whether albumin in the G-A animal influenced the globulin antibody directly or indirectly after formation of albumin antibody. Five animals were available which had been injected with globulin 19 days previously; an injection of albumin was given and the animals were tested after a further 10 days. Globulin, 0·15 mg./100 g., was injected into the mesenteric vein, the first horn was removed, and 0·35 mg./ 100 g. was injected. One and a half hours later globulin was given to bring the total to 1·5 mg./100 g. When the animals were killed after the usual period the uterine horns of two were found to be desensitized. The result was inconclusive, and a larger number would be required to settle the point: no sign of shock was observed in any of these animals.

The converse of the above type was examined, animals which were given an injection of albumin and after 31 days were injected with globulin (A-G animals): these were killed 17–20 days after the globulin had been given. The first uterine horns of six were removed before any globulin was given into the mesenteric vein, and were tested with 1 mg. of albumin, then with an additional 1 mg. of albumin; the Ringer's solution was changed and 1 mg. of globulin was placed in the bath, followed by 5 mg. Two uteri were insensitive to albumin and to globulin: the remaining four responded by maximal contractions when 1 mg. of albumin was introduced; a further 1 mg. of albumin caused no reaction, nor did globulin. The whole of the sensitivity of these uteri was thus discharged by a small amount of albumin. The remaining five animals received a small injection of globulin before the first horns were removed, since the A-G animals seemed to be even more sensitive to globulin than was the G-A series: the uteri, however, all responded to albumin, none to globulin. None of the isolated uteri of these animals was tested with globulin before introduction of albumin into the bath, since it was vital to be certain of the sensitivity of these uteri to albumin, and thus to differentiate them from the uteri of animals sensitized by serum, synthetic serum, and globulin followed by albumin. The results of the injection experiments are given in Table 26. In two of the animals, death in typical anaphylactic shock occurred about 5 min. after an injection of globulin. Three others, which showed no immediate reaction to the injected protein and which made good recovery from the operation, gradually collapsed and died $\frac{1}{2}$–$1\frac{1}{4}$ hr. later. Only four lived for the standard period of $1\frac{1}{2}$–2 hr., and two of these animals were noticeably weak. Globulin appears to cause delayed and fatal shock in the A-G animal, while it caused no symptoms of any kind when injected into the animal sensitized by albumin alone. This fact of itself is sufficient to indicate that, though the uteri of the A-G series are highly sensitized to albumin, the animals are quite different from albumin-sensitized animals. Also, all the second uterine horns which were examined were found to have

The very large difference in the time of grinding in our experiments and those of Cohen, suggests that the extent of the denaturation of the albumin and globulin from this cause must have been very small. Appreciable denaturation would reduce the value of conclusions drawn from the difference found between serum and the proteins derived from it, when injected into the circulation. On the other hand, denaturation would enhance this difference when the intestinal route was being used; under these conditions serum was shown to be much less active than albumin and globulin.

The primary purpose of this work, the decision as to whether the smaller molecule albumin is absorbed from the intestine more readily than globulin, has not been achieved. The unexpected finding that globulin may be absorbed, probably by the lymphatic system, renders valueless any calculation based on the relative amounts of the two proteins which are required to desensitize the animal when injected into the portal blood. There is no reason why albumin itself may not enter the lacteals after leaving the lumen of the intestine; but there is not even indirect evidence that this may occur, as there is in the case of globulin. All that can be said, until further information is available, is that albumin and globulin, or proteins antigenically indistinguishable from them, are absorbed in significant amounts within a few hours of their introduction into the small intestine. Such absorption is probably not limited to the artificial conditions which obtained in this work, and it may well occur normally if some protein escapes the action of the proteolytic enzymes in the digestive tract. There was no significant distension of the gut when the albumin and globulin were injected, since the volume of fluid did not exceed 1·0 c.c. When serum was used to desensitize the animals sensitized by this protein, desensitization was only effected by an amount so large that it was impossible to avoid marked distension of the duodenum. Since the gut was not ligatured the distension would be gradually relieved as the serum distributed itself, but it is impossible to be certain how far the distension factor came into play, a factor which Hettwer & Kriz [1925] have shown to affect the absorption of serum from the intestine.

The fact that some sensitized animals died in anaphylactic shock, while others, receiving the same sensitizing treatment and reinjection, showed no symptoms, is not easy to explain. In the animal sensitized to albumin and desensitized by a single injection of this protein, the antigen-antibody reaction must be a comparatively mild event in the cells, and, if it involves some cellular injury with liberation of histamine, the amount so liberated must be small. When such animals were desensitized by serum or synthetic serum a much more drastic event occurred, and there was always the risk of death, even when the antigen was given in graded amounts. Other examples of highly toxic reactions have been given in the text and need not be repeated here. It is remarkable that it was possible, on occasion, to desensitize an

of killing should be correct for both the milk test and the anaphylactic work. The high degree of species specificity of the anaphylactic reaction precluded any interference by the proteins of milk, but the validity of results obtained from animals in which tuberculosis was active was open to doubt. After consideration of a long series of experiments carried out on the milk-test animals, it has been decided not to exclude results from animals which had been infected by the milk. All the animals used appeared healthy when the operations were performed, and only when the records of the Department of Bacteriology were consulted, at intervals of about 6 months, were the results of the milk tests known. On looking back through the work performed during such a period, it was not possible to detect any anomalous results which could be attributed to the presence of tuberculosis. The disease did not significantly affect the capacity of the animals to become sensitized to horse protein. Of the 368 animals which were sensitized, 37 or 10·1 % were tuberculous: of the 297 animals whose first uterine horns proved to be insensitive, 26 or 8·7 % had tubercular lesions. The average weight of the milk-test animals was 368 g. These animals were not necessarily virgin, and some were found to be pregnant when the abdomen was opened: if pregnancy was not advanced these uteri were used, many with success, since, if the strip of muscle relaxed to give a steady series of uniform small contractions, the large response which followed the introduction of the antigen into the bath was as clearly marked as in the case of a small virgin uterus.

In addition to the milk-test animals, 132 small virgin guinea-pigs were specially obtained for the anaphylactic experiments alone; their average weight was 298 g. These animals were used late in the work, not because of any doubt as to the validity of the other results, but to supplement the supply of animals from the Department of Bacteriology at a time when it was inadequate, to confirm results which differed from those of other workers, and when it was necessary to sensitize some animals for longer than 4–5 weeks.

After the experiments were completed, there appeared an important paper by Cohen [1943], on the effect of dry grinding on the properties of proteins. Cohen, working with ovalbumin, showed that grinding the protein in a dry state for 24 hr. or longer, produced changes in the molecule associated with denaturation and coagulation. It seems possible that some alteration in the proteins may have occurred during the grinding of the albumin and globulin used in our experiments; the time of grinding was not controlled, and doubtless varied in different preparations, but it was never prolonged. When the work of Cohen was known, the time of grinding of three globulin preparations was accurately noted; globulin was chosen, since this protein becomes a hard glass involving more grinding than albumin. The longest time taken to reduce the protein to the usual degree of fineness was 1 min. 25 sec., and it is doubtful whether any preparation used in this work was ground for more than 2 min.

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animal by a full dose of the appropriate antigen without shock, while other animals of the same series showed symptoms or died after only a minute dose. The first uterine horns of the animals which were desensitized without shock were often as sensitive as those of animals to which a much smaller dose of antigen proved fatal. It is possible that the reaction of certain sensitized cells in the liver may afford an explanation. The possibility of the active participation in anaphylactic shock in the guinea-pig of sensitized cells elsewhere than in the lungs was considered by Dale [1912], who concluded that there was no necessity to assume any other than the immediate effect of the antigen on the sensitized muscle in the lungs, to explain acute shock. Numerous instances have been given in the present work of sudden and rapidly fatal shock in some sensitized guinea-pigs, particularly in those sensitized to globulin, which follows injection of the antigen into the portal blood, without accompanying desensitization of the uterus; it is probable therefore that the liver may play an important part in some acute anaphylactic reactions in these animals. When fatal shock occurred in 3–5 min. after injection of an antigen into the portal blood, and the uterus was found to be fully sensitive, very little, if any, of the foreign protein could have entered the systemic circulation, and death must have been caused indirectly: the spasm of the bronchial musculature, which is usually considered to be the immediate cause of death in anaphylactic shock in the guinea-pig, must have been secondary to the liberation from the liver of some non-antigenic stimulant of smooth muscle, such as histamine. It is possible therefore that when animals are sensitized by a protein, receptive cells in some tissues, e.g. the liver, are more highly sensitized than in others, and that this differential distribution of the sensitizing antibody varies from one individual to another. This may account for the death in shock of some animals given a very small amount of protein, by the portal circulation, and the absence of any symptoms in others, of apparently equal uterine sensitivity, which received a far larger quantity of the antigen. Protein introduced into the intestine enters the circulation so slowly as to desensitize even the most highly sensitive cells without symptoms, for none have been noted in all the animals desensitized by this route.

It is known that the epithelial cells of the intestinal mucosa discriminate sharply between substances of similar physical properties, and the failure to correlate, in the three types of sensitized animals studied, the desensitizing power of serum with that of albumin and globulin, is a point in favour of serum containing a complex protein in which albumin and globulin have no separate existence. The properties of serum as a desensitizing antigen, when injected into the portal blood, agree closely with those found for an artificial mixture of albumin and globulin, with the curious exception that serum was less consistent in its action than was 'synthetic serum' injected into the globulin animal. Globulin alone was also more reliable than serum in desensitizing

the animal sensitized by the latter. A mixture of albumin and globulin injected into the blood was found to desensitize in circumstances which could not be explained as the action of either of the proteins alone, and it is difficult to account for this in any other way than as due to some type of recombination between them. Macfarlane [1935] has shown that mixtures of albumin and globulin show changes in the ratio of large to small molecules, which must be due to some·interaction. If protein complexes exist in serum and in mixtures of albumin and globulin, they are unlikely to be identical, since definite differences were found between serum and 'synthetic serum' when acting as desensitizing antigens. There was also a difference between them when acting as sensitizing antigens. The most cogent evidence in favour of serum containing a complex protein was afforded by the special properties of the guinea-pig which had been sensitized by serum: these were not always reproduced when 'synthetic serum' was used, nor were they always exhibited after sensitization by prediluted serum. The evidence that serum is unique as a sensitizing complex is essentially based on the influence of the 'masked' albumin. an influence which leaves the uterine horns unresponsive to albumin *in vitro*, but which makes the serum-sensitized animals differ completely from those sensitized to globulin, and remain so for at least 35 days. That the serum antibody should show evidence of breakdown between the 35th and 50th days is not incompatible with serum being an albumin-globulin complex, for this must be of the loosest possible kind: it is reasonable to suppose that the antibody may be dissociated ultimately by factors in the cells, just as the antigen which gave rise to it is readily dissociated by water or salt solutions. The alteration which takes place in the serum antibody is compatible with waning influence of the albumin component, which results in the animal becoming more like one sensitized to globulin. A similar change takes place in the animals sensitized by synthetic serum, but at an earlier date. Since nothing is known of the composition of these antibodies, it is not profitable to speculate further, but the results of the experiments with the G-A and A-G animals suggest that there may be fixed in the cells of different animals more than one type of antibody, in the formation of which both albumin and globulin have taken part. An animal sensitized by a single protein can have *only* one type of antibody present in the cells, and this should remain unchanged, qualitatively, until it disappears in the course of time; when an antibody complex is formed as the result of the injection of two proteins, the components need not necessarily persist with the potency of each unchanged over a long period; one may diminish in strength before the other.

The non-homogeneity of serum shown by Macfarlane [1935] in his ultra-centrifuge experiments is not incompatible with serum containing a protein complex. As Macfarlane stated, there was no evidence that the large particles are globulin, and it is possible that there may be more than one type of protein

complex in serum. If albumin and globulin associate together, complexes may be formed in which the constituent proteins are present in different proportions, thus giving rise to particles of different size. Other difficulties are the change from normal of the albumin/globulin ratio in pathological sera and the analytical figures which show different rates of regeneration of these proteins in the blood after haemorrhage. It is possible that serum protein is not of fixed and invariable composition, and that it may be made up of different proportions of the constituent proteins, depending upon the conditions. It is not easy to see how a final answer to this question may be given from the very nature of the problem the evidence must be indirect. All that can be said at the present time is that serum, examined by different observers and different methods, does not behave merely like a solution of free albumin and free globulin.

SUMMARY OF PART 2

1. Considerable quantities of globulin have been administered by the intestinal and portal routes to guinea-pigs sensitized by albumin. Cross-reaction did not occur, nor were they observed when the converse experiments were performed.

2. Though the amount of globulin required to desensitize a globulin sensitive animal by the portal route was large, a very small quantity of globulin was found to be effective when an equal amount of albumin was present ('synthetic serum'). The volume of natural serum required to desensitize such an animal was also very small.

3. The characteristics of serum' and 'synthetic serum' as desensitizing antigens have been studied and compared. 'Synthetic serum' simulates serum closely in some reactions, but not in all.

4. The influence of serum as a sensitizing antigen has been investigated. Animals sensitized by serum for 30 days differ from some of those sensitized by diluted serum or by a mixture of albumin and globulin.

5. Animals which were sensitized by serum for 50 days differed from those which were examined after 30 days.

6. The behaviour of serum as a desensitizing and as a sensitizing antigen is incompatible with that of a mixture of albumin and globulin, and it is suggested that serum contains a protein complex in which albumin and globulin have no separate existence.

7. The isolated uteri of animals sensitized by serum, 'synthetic serum', or globulin followed at 3 weeks' interval by albumin are not responsive to albumin. The uteri of animals sensitized by albumin followed by globulin after 31 days are very sensitive to albumin *in vitro*.

8. It is shown that more than one type of antibody may exist, in the formation of which both albumin and globulin have taken part.

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A STUDY OF DEHYDRATION BY MEANS OF BALANCE EXPERIMENTS

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A survey of the literature purporting to deal with the effects of water deprivation shows that most of it has really been concerned with the results of a deficiency of salt. In 1935, however, Kerpel-Fronius distinguished between the changes in the body due solely to a deficiency of salt, and those due simply to a shortage of water, but this important distinction has been little appreciated either in this country or in America, although Nadal, Pedersen & Maddock [1941] set out to contrast the features of these two syndromes in man. Unfortunately, in one of Nadal *et al.* two subjects the period of water deprivation followed directly upon one of salt deficiency, while in the other deprivation of water was accompanied by fasting. Hence, although results of great interest were obtained, the effects of water deprivation were complicated in one person by salt deficiency, and in the other by fasting. Admittedly, in clinical medicine, deprivation of water is usually accompanied by one or other of these complications, but warfare by sea or land may subject men with plenty of food to a shortage of water.

The present paper describes the results of metabolism studies on two subjects, who abstained from water for 3 and 4 days respectively, during which they took a 'dry' diet adequate in protein, salt and calories. Although a balance experiment on a human being cannot be carried to the point where the biochemical cause of death becomes clearly stigmatized, it is possible to distinguish physico-chemical happenings in the early days of water deprivation which would, if they went on long enough, provide a reasonable *modus moriendi*. It is this chain of events which it is now proposed to describe.

METHODS

A preliminary experiment was carried out on R. M. with the object of studying the general features and particularly the function of the kidney during dehydration. This made it clear that some people would certainly be able to

maintain a normal calorie intake for the first few days of dehydration. D. B. and J. H., two normal men aged 28 and 33, and weighing 63 and 70 kg., were then studied in the following way. Each subject selected his own diet with a view to its palatability without water, and this diet was kept constant in weight and composition from day to day. D. B.'s diet comprised brown bread, butter and marmalade, cheese, dates, meat paste, shortbread, oatcakes and toffee. It contained 2·9 g. of K, 9·1 g. of NaCl, and 13·8 g. of N per day, 470 g. of carbohydrate, 80 g. of protein. 115 g. of fat, and its calorie value was 3164. J. H.'s diet consisted of biscuits of various types, butter and jam, cheese, bacon, potato crisps, chocolate and toffee. It yielded 1·8 g. of K, 9·6 g. of NaCl, and 10·4 g. of N per day, 286 g. of carbohydrate, 64 g. of protein, 161 g. of fat, and its calorie value was 2846. D. B.'s daily food contained 251 c.c. of water, and a further 439 c.c. of water would have been derived from its complete metabolism. For J. H. the corresponding figures were 115 and 359 c.c. For 4 days each person took his diet with as much water, tea or coffee as he felt inclined. All fluids were then withheld for 3 days from D. B., and for 4 days from J. H. A further period of 3 days with fluids concluded each experiment. Both men were weighed every day. Urine and faeces were collected for 2 days before fluids were withdrawn and thereafter to the end of the experiment. Urine was collected under toluol in 24 hr. periods; faeces were collected as described by McCance & Widdowson [1942]. Blood was always withdrawn at the same time of day. Chlorides in the plasma were determined by Sendroy's [1937] method, and, apart from this, urea and the mineral elements by the methods given by McCance [1937] and McCance & Widdowson [1937].

<center>RESULTS</center>

<center>*General changes*</center>

In addition to the subjects described in this paper there have been opportunities for observing the general effects of experimental dehydration in eight men and two women. The experience of voluntary dehydration is inseparable from some degree of 'self consciousness'. Even allowing for this, the subjects exhibited a change in behaviour which could be interpreted as an exaggeration of their temperamental type. Serious people became positively sombre; while others, normally cheerful, exhibited a somewhat hollow vivacity. The subjects were intellectually capable of performing estimations and calculations, but their concentration was impaired. They found that the days of dehydration were not actually uncomfortable but they seemed very long. The experimenters were never unbearably thirsty, but by the third day their mouths and throats had become dry, their voices husky, and they had begun to find it difficult to swallow. By the third or fourth day their facies had become somewhat pinched and pale and there was a suggestion of cyanosis about their lips

which was rather characteristic. This general appearance of ill-being vanished within a few hours of the restoration of fluid, and the symptoms of dehydration passed off long before physiological rehydration was complete. Many of the subjects lost all their desire to drink as soon as the first pint of fluid had been taken. Both D. B. and J. H. were rehydrated slowly [McCance & Young, 1944], and this should be borne in mind when considering the metabolic changes of the first day of rehydration. The subjects lost weight at rather different rates. D. B., for example, lost 3·8 kg. in 3 days, and J. H. 3·8 kg. in 4 days.

Changes in the circulating fluids

The haemoglobin and haematocrit values changed very little, and there was no evidence that either of them rose during the periods of dehydration. D. B.'s plasma proteins increased from 7·2 to 7·4 % and J. H.'s from 7·3 to 7·6 %, and, although they fell again during the after periods, it is doubtful if these changes have much significance. It may be inferred that under the conditions of these experiments there was little if any reduction in the blood volume. These findings are in accordance with those of Nadal *et al.* [1941] and demonstrate that some highly efficient mechanism must have been at work to prevent such a reduction taking place, for, at the same time, the volume of other fluid compartments of the body measurably declined.

TABLE 1. Changes in the serum induced by dehydration

Subject	Last day of	Plasma Na mg. per 100 c.c.	Serum K mg. per 100 c.c.	Plasma Cl mg. per 100 c.c.	Plasma urea mg. per 100 c.c.
D. B.	Fore period	340	19·3	377	33
	Dehydration period	369	15·1	406	57
	Rehydration period	340	16·6	374	29
J. H.	Fore period	327	15·6	364	20
	Dehydration period	356	13·2	384	47
	Rehydration period	334	13·5	361	27

The changes in the serum chemistry are given in Table 1. The blood urea always underwent a moderate increase during the dehydration period. The figures in Table 1 may be regarded as fairly typical, although greater increments have been recorded in other subjects. The plasma Na rose steadily in the dehydration period to a value about 10 % in excess of its initial one, and fell to its original level after rehydration. There were similar changes in the plasma Cl. There was a fall in the serum K of both men during the dehydration period. This is particularly noteworthy when it is recalled that there was an increased excretion of K in the urine at this time [McCance & Young, 1944].

Nitrogen metabolism

The N balances of both men are given in Table 2. Since D. B. was in positive and J. H. in negative balance during the two preliminary days, it is difficult in some ways to compare the two experiments. Both subjects, however

TABLE 2. Nitrogen balances before, during and after dehydration

	D. B.			J. H.		
Day	Intake g. per day	Total output g. per day	Balance g. per day	Intake g. per day	Total output g. per day	Balance g. per day
Preliminary (average)	13·5	12·3	+1·2	10·4	11·3	−0·9
Dehydration 1	14·0	10·0	+4·0	10·4	10·3	+0·1
2	14·0	12·6	+1·4	10·4	14·3	−3·9
3	14·0	14·4	−0·4	10·4	15·3	−4·9
4	—	—	—	10·4	16·1	−5·7
Rehydration 1	13·9	17·0	−3·1	10·4	15·1	−4·7
2	13·9	17·5	−3·6	10·4	17·6	−7·2
3	13·9	13·4	+0·5	10·4	14·4	−4·0

howed a fall in the excretion of N on the first day of dehydration, during which most of the rise in the blood urea took place, and an increased elimination of N during those parts of the after period when the blood urea was regaining its original level. In order to assess the daily formation of urea, these changes in the levels of urea in the body fluids had to be taken into account, as well as the urinary excretion, and when this was done the figures shown in Table 3 were obtained. It will be seen that the urea production

TABLE 3. The effect of dehydration on the production of urea

	D. B.	J. H.
Average preliminary period g. per day	19·5	14·9
Average dehydration period g. per day	22·8	20·6
Average rehydration period g. per day	21·8	18·0

increased during the dehydration period and remained somewhat high into the after periods. This increase was greater in J. H. than in D. B., but in both it was greater than could be accounted for by dietary variation or experimental inaccuracy. It may be attributed to a breakdown of tissue protein. In one sense the quantities involved were small, for they amounted, even in the case of J. H., to less than 20 g. of body protein per day. Had the dehydration been prolonged, however, and the losses continued, they might later have become a serious matter.

The water balance

When Newburgh & Johnston's [1942] method of calculating changes in the body water was applied, the figures given in Table 4 were obtained. These findings agree quite well with those of Coller & Maddock [1935], whose two subjects, studied under similar conditions, also lost about 3 kg. of water in 3 days. The loss of water by the skin and lungs tended to decrease during the dehydration period. This may have been due to diminishing activity, for Newburgh & Johnston [1942] have shown that when they controlled the activity of a subject his insensible water loss did not diminish during dehydration of a similar degree. Neither subject can have been losing much water or

TABLE 4. The water balances during the periods of dehydration

Subject and duration of dehydration	Intake c.c.			Output o.o.			Balance c.c.
	In food	By metabolism	Total	Urine	Lungs and skin	Total	
D. B. (3 days)	753	1287	2040	1933	3637	5570	−3530
J. H. (4 days)	460	1436	1896	2360	3066	5426	−3530

NaCl by the active production of sweat for it is accepted that the water lost by the insensible perspiration of the skin and by the lungs is usually of the order of 1000 c.c. per day. This conclusion is important in connexion with the mineral balances now to be considered.

Mineral balances

The figures obtained from D. B. were more satisfactory than those from J. H. for D. B. was in mineral balance during the preliminary period, while J. H. was gaining slightly in weight and was in slightly positive Na and Cl and in negative K balance. Nevertheless, the subjects agreed in showing a small retention of Na and Cl during and after dehydration and a negative K balance during dehydration. Similar results were obtained by Wiley & Wiley [1933], who studied the mineral balances of a man whose water intake was restricted to 1185 g. per day for a period of 6 days. The present results are summarized in Table 5. The output by the faeces was measured and taken into account,

TABLE 5. The mineral balances

Average for:	D. B. (3 days dehydration)			J. H. (4 days dehydration)		
	Na g. per day	Cl g. per day	K g. per day	Na g. per day	Cl g. per day	K g. per day
Preliminary period	±0·0	±0·0	±0·0	+0·7	+1·3	−0·7
Dehydration period	+0·5	+0·7	−1·1	+0·6	+2·4	−1·0
Rehydration period	+1·1	+2·0	+0·5	+1·2	+0·7	+0·1

but not the losses on the surface of the body. The figures indicate that these men did not excrete quite all the salt in their diets while they were being dehydrated and none of the salt from their extracellular fluids. Since these fluids underwent a considerable shrinkage in volume before the dehydration was relieved, it is easy to understand why the serum Na was observed to rise (Table 1). In the experiment of Nadal et al. [1941] the diet was salt free and the Na and Cl balances were, therefore, negative. In 3 days of dehydration, however, their subject lost less than 1 g. of Na, which would correspond roughly to 300 c.c. of extracellular fluid. He must have lost much more of his extracellular fluids than this—perhaps in all as much as 1000 c.c.—and retained the equivalent amount, about 2 g., of Na. Similar happenings were observed when dogs were deprived of water [Elkington & Taffel, 1942] and are likely to be found in all mammals. The retention of Na and Cl in the after period was part of the reaction of the body to rehydration. During this time drinking water

was diluting the body fluids and (for some reason) Na and Cl were retained concurrently.

As already mentioned, both men had a negative K balance while they were being dehydrated. D. B. excreted 3·28 g. of K over and above the amount in his food. This corresponds to about 600 c.c. of cell fluid, and J. H. excreted in the same way K corresponding to about 740 c.c. of cell fluid. It is interesting to compare these volumes with those corresponding to the amounts of tissue being broken down, as estimated from the increased urea production during the dehydration periods. In D. B. 10 g. of 'extra' urea were formed during dehydration (Table 3) and this is equivalent to 30 g. of protein. Protein is associated with three to five times its own weight of water in the tissues [Best & Taylor, 1939] so that only about 125 c.c. of intracellular water were set free by the destruction of protein. Similarly in J. H. the 'extra' urea formed during dehydration had a protein equivalent of 70 g. corresponding to about 300 c.c. of cell water. In passing it is to be noted that Kerpel-Fronius [1935] found that when fasting rabbits were deprived of water they too excreted a higher K/N ratio than is present in cell fluid. D. B. and J. H. both lost over 3000 c.c. of body fluids during dehydration and of these at least 2000 c.c. probably came from the cells. Hence during dehydration the happenings in the cells may be summarized as follows: (*a*) There was a considerable loss of fluid. (*b*) There was a loss—by excretion—of *some* of the K corresponding to this fluid but nevertheless a rise of osmotic pressure within the cells. (c) There was a loss—by destruction—of *some* of the cell protein, but not enough to correspond to the loss of K. Hence during dehydration the cells become smaller, more concentrated with respect to K ions and still more concentrated with respect to protein.

The positive Na and negative K balances during the dehydration periods show that K from the cellular fluids had priority in excretion over Na from the extracellular fluids, and this in spite of the fact that the K in the plasma fell during dehydration while the Na in the plasma rose. This preferential excretion of K was noted and discussed by Elkington & Taffel [1942]. These authors pointed out that by this means more water was withdrawn from the intracellular compartments of the body than would otherwise have been the case, and hence that the volume of the extracellular fluids was to the same extent preserved. This may help to explain the maintenance of the blood volume, previously described, and commented upon also by Nadal *et al.* [1941].

It is instructive in summary to contrast the course of events in dehydration and in salt deficiency. In the former the excretion of minerals did not keep pace with that of water, the osmotic pressure of the plasma and of the whole body rose, some K passed out of the cells, and this helped to preserve the volume of the extracellular fluids. The plasma volume did not fall. In salt deficiency [McCance, 1936; McCance & Widdowson, 1938] the excretion of

water did not keep up with the loss of salt and consequently the osmotic pressure of the plasma fell. Water passed into the cells and there was a great fall in the volume of the extracellular fluids. The volume of the plasma and of the circulating blood fell also, and this was probably the cause of much of the subjects' embarrassment and discomfort.

The osmotic balances

D. B.'s diet contained 155 m.eq. of Na, 155 m.eq. of Cl, 74 m.eq. of K and enough protein to produce 325 mM. of urea, giving a total of 709 mM. of osmotically active material per day. J. H.'s diet contained 631 mM. of these four substances per day. Table 6 shows the amounts of osmotically active material

TABLE 6. The osmotic balances

	Subject D. B.		Subject J. H.	
	Diet	Urine	Diet	Urine
Period and day of period	mM. per day	mM. per day	mM. per day	mM. per day
Preliminary (average)	710	702	631	614
Dehydration 1	710	577	631	528
2	710	702	631	666
3	710	754	631	676
4	—	—	631	722
Rehydration 1	710	669	631	528
2	710	628	631	538
3	710	697	631	626

(as represented by the Na, K, Cl and urea) excreted by both men. It will be seen that the osmotic excretion in the urine was low on the first day of dehydration, and later rose, so that by the last day the kidneys were excreting more mM. of the four substances together than the diet provided, in spite of the small volume of water available to them [McCance & Young, 1944]. These daily fluctuations in osmotic output were due to urea (see Table 2) and not to the minerals. Over the whole dehydration periods the kidneys may be said to have excreted the osmotically active material in the diet, and, but for the changes in body water, the men would have been in osmotic equilibrium. In the same period, however, 3·5 l. of body fluids were lost, and each of these would have contained about 320 mM. of osmotically active material, making a total of about 1100 mM., which were either retained or, more probably, partly excreted with the retention of an equivalent amount of osmotically active material from the food. This retention corresponds to an increase of about 28 m.osmol./l. in the 40 l. (approx.) of body fluids. The percentage of Na in the plasma increased by about 30 mg./100 c.c., or about 13 m.eq./l. There must have been an equivalent increase in acid radicles and a small change due to urea—say 30 m.osmol. in all. There is thus good agreement between two very different methods of estimating the increase in the osmotic pressure of the body.

It might quite reasonably be supposed that if the kidneys were able to excrete 600 to 700 mM. of osmotically active material per day on a diet containing just that amount, they might do so on a diet poorer in salt and protein so that on such a diet the loss of body fluid by dehydration might not involve any increase in the osmotic pressure of the body. The results of Nadal *et al.* [1941] show, however, that even with a fasting subject the excretion of minerals derived from the mobilization of body water is not complete. In their experiment IIB, the losses of minerals and urea in the urine during a 3-day period of dehydration were: Na, 41 m.eq., Cl, 59 m.eq., K, 150 m.eq. and urea 93 mM.—a total of 343 m.osmol. Since no food was taken, all this was derived from the body fluids, but in the same period there was a loss of 3264 c.c., which would have contained 1045 m.osmol. Even in complete fasting, therefore, there was a retention of 702 m.osmol. in 3 days. Other aspects of this phenomenon are discussed by McCance & Young [1944].

This osmotic retention, which appears to be somewhat greater on a full diet than in fasting, must be seriously considered as the cause of death in dehydration. Kerpel-Fronius & Leövey [1929] found that after ligating the ureters the osmotic pressure of both the blood and the tissues tended to rise, and that death could be hastened by giving either salt or urea. They concluded that the rise in the total osmotic pressure of the body and not the accumulation of any specific substance was the cause of death. Kerpel-Fronius & Leövey [1931] later found a similar increase in the osmotic pressure of the blood and tissues in experimental dehydration in puppies, and suggested that here too death might be due to the same disturbance in the osmotic pressure of the tissues. Elkington & Taffel's [1942] observations on dehydrated dogs lend some support to this suggestion, for the increase in the total ionic concentration of the body fluids was greatest in the animals which died or became moribund. It would be unreasonable to expect absolutely definite indications of the cause of death in dehydration from experiments such as these lasting but 3 or 4 days, since it is known that men may survive on very small amounts of water for several weeks. Within the limits imposed by human experiments, however, the present findings support the conclusions of Kerpel-Fronius that death from gradual dehydration may be due to the increased osmotic pressure of the body.

SUMMARY

1. Balance experiments have been carried out on two men before, during and after a period of experimental dehydration.

2. During dehydration there was no reduction in the plasma volume. The serum Na rose and the serum K fell.

3. Dehydration increased the amount of urea produced by the body.

4. The men lost over 3500 c.c. of their body water, but none of the Na from

their extracellular fluids and only a little of the K from their cellula
fluids.

5. The osmotic pressure of the body rose, and it is considered that this ma
be the cause of death in dehydration.

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THE SECRETION OF URINE DURING DEHYDRATION AND REHYDRATION

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Functional studies of the kidney have usually been made while subjects have been passing large volumes of urine. Admittedly, the general characteristics of urine passed during short periods of water restriction are well known, and a number of clinical tests have been devised based upon the capacity of a person when so restricted to produce a concentrated urine. On the whole, these tests have not contributed much to our knowledge of renal physiology and will not be discussed. A few people, however [Adolph, 1921, 1923; Coller & Maddock, 1933; Chesley, 1938; Nadal, Pedersen & Maddock, 1941], have braved the difficulties and planned experiments which allowed them to investigate the secretion of urine when the intake of water was low. The knowledge accumulated by these and other workers may be summarized as follows.

If in a temperate climate the total intake of water, including water of metabolism, is less than 1500 c.c./day, the volume of urine is small, and it is highly concentrated, but even in complete water deprivation some urine is secreted. This has a specific gravity of over 1030 if the concentrating ability of the kidney is normal, and it contains 6–8 g. of solids per 100 c.c. [Lashmet & Newburgh, 1932]. Coller & Maddock [1935] considered that the total solids to be excreted were of the order of 35–40 g./day, and that, therefore, 500 c.c. of urine per day were needed to carry off the waste products of the body. Chesley [1938] found that below a critical volume of 21–30 c.c./hr. the total solids were always concentrated by the kidney to the same extent, which was the maximum extent of which that kidney was capable, so that the amount of them excreted depended mainly upon the volume of the urine. He also showed that below this critical volume the concentrations of urea and endogenous creatinine were always the same, and he therefore held that the clearances of these substances varied exactly with the output of water at these low volumes. He considered that his findings demonstrated a direct relationship between the glomerular filtration rate and the rate of urine flow,

but he did not measure the filtration rate by estimating the inulin clearances. There were no records of this having been done in dehydrated persons, but Shannon [1936] has estimated the creatinine clearances in dogs at very low rates of urine flow. In this species the creatinine and inulin clearances have the same value, and Shannon found that there was a tendency for these clearances to fall when the minute volume of the urine was reduced below 0·5 c.c. but not by more than 10–20%. If the animal had been deprived of water for 24 hr. and had become dehydrated, the creatinine clearances were sometimes lower than they had been earlier in the experiment at comparable rates of urine flow.

The present investigation was prompted by the fact that the vicissitudes of war were forcing people to live and work in places where they were extremely short of water. Little was known about the way in which the healthy kidney reacted to dehydration, and, since this was a matter of cardinal importance, it was decided to study it. To do so, normal men and women were dehydrated experimentally by depriving them of water, and the functions of their kidneys were studied in various ways and under varying degrees of bodily hydration. Some of the experiments have embraced preliminary days on a standardized diet, 3 or 4 days of dehydration, and a period of rehydration. They have been designed to study the effects of food, water and salt upon the daily output of the kidney and they therefore necessarily involved the continuous collection of the excreta. Other experiments, planned for the study of glomerular filtration rates or to follow the effects of large doses of urea, Na and K salts, have lasted only a few hours, and, except for the dehydrating diet, the subjects have not necessarily been burdened with much experimental control except at these times.

SUBJECT AND METHODS

In all, twelve persons have been dehydrated, ten men and two women. Their ages have ranged from 20 to 43. The usual practice has been for them to drink no water for 84, or in one case 108 hr., and to eat only dry food such as biscuits, chocolate and potato crisps, but the dietary ingredients have varied with the dictates of the particular study. Two of the metabolism experiments have been described in some detail [Black, McCance & Young, 1944]: the necessary information about the others will be given when the results are being set out.

Inulin was determined both in urine and plasma by an unpublished method devised by S. W. Cole. This depends upon the colour which develops when fructose, HCl and resorcinol are heated together under standard conditions. Resorcinol is a more satisfactory and sensitive reagent than diphenylamine. The intensity of the colour was measured in a photoelectric colorimeter. Diodone was determined by Alpert's [1941] method. For the measurement

of the glomerular filtration rates and renal plasma flows in the hydrated subjects, 3·0 g. of inulin and 2·0 c.c. of diodone were injected intravenously as a priming solution [Smith, Goldring & Chasis, 1938] in 2·5 c.c. of 'normal' saline. This was followed by a maintenance infusion which contained 4·0 g. of inulin and 6·0 c.c. of diodone in 50 c.c. of 'normal' saline. In all these experiments the subjects emptied their bladders after the maintenance infusion had been running for about 20 min. The exact time was noted, and then at intervals of about 30 min. the subjects emptied their bladders voluntarily till two or three samples had been collected. They found no difficulty in doing this. To assist them the maintenance infusion was stopped for 1 or 2 min. and the subjects voided their urine standing in the natural position. Men only were used for these experiments. The blood for analysis was taken by vein puncture midway between one urine collection and the next.

Urine samples were preserved with toluene. 'weighed solids' were determined by weighing the residue after 5 c.c. of urine had been dried overnight at 93° C. The same pipette was always used for these measurements. The chlorides in the plasma were determined by Sendroy's [1937] iodine titration method. Chlorides in the urine, urea, Na, K, phosphates and other radicles were determined by the methods given by McCance [1937] and McCance & Widdowson [1937].

PRESENTATION AND DISCUSSION OF THE RESULTS

The inulin and diodone clearances

These results have already been reported in a brief note [Black, McCance & Young, 1942].* It is only necessary here to state that dehydration, uncomplicated by starvation but sufficiently severe to make the subjects lose 4–7 % of their body weight, slightly reduced the inulin clearances of three subjects and made no difference to a fourth. At the same time dehydration appeared to increase the diodone clearances of three of the men and to bring about a small fall in one. It is evident therefore that the glomerular filtration rates of these men, and also their renal plasma flows, were little changed by their dehydration. These are important points, not only in themselves but because they mean that the changes which characterize the urine during dehydration of this severity must be essentially the result of alterations in tubular activity.

The effects of large doses of NaCl, KCl and urea

In general, all these experiments were carried out in the same way. On the morning after a normal evening meal and night, a strong solution of the salt was administered, and the effect upon the volume of the urine and upon the composition of the plasma was followed during the next few hours. The subject was then dehydrated for 3 days in the way already described, the salt solution was given and, as before, the plasma and urine were collected

periodically for some hours. Somewhat similar experiments were carried out by Adolph [1921], but at that time no samples of blood appear to have been taken for analysis, and very little data about the urine were given.

(a) NaCl. Two persons were used for these tests and both responded in the same way, so the results for only one of them will be described. Miss H. drank 500 c.c. of a solution containing 18 g. NaCl on each occasion and a summary of the findings is given in Table 1. It will be noted that the effect

TABLE 1. The effect of taking 500 o.o. of 3·6 % NaCl on the secretion of urine during dehydration

Time	Urine volume c.c./min.		Urine urea m.mol./l.		Urine Na m.eq./l.		Urine K m.eq./l.		U/P osmotic ratio*	
	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated
Before salt	0·46	0·36	490	588	148	205	136	59	3·10	4·21
After salt 1	1·93	1·56	178	280	249	305	69	51	2·61	3·23
After salt 2	1·57	1·65	213	235	260	322	97	36	2·77	3·36

$$* \text{ U/P ratio} = \frac{\text{m.mol. of urea} + 2 \text{ (m.eq. of Na} + \text{K)} \text{ in the urine}}{\text{m.mol. of urea} + 2 \text{ (m.eq. of Na} + \text{K)} \text{ in the plasma}}.$$

of the salt solution was essentially the same when she was normal and dehydrated and consisted in: (1) A diuresis. (2) A fall in the concentration of urea in the urine. This was partly a dilution effect due to the forced diuresis. From the data given, however, it will be clear that there was a rise in the output of urea per minute, the result presumably of the increased output of water, for it is well known that the magnitude of the urea clearance depends upon the rate of excretion of water. (3) A rise in the concentration of Na in the urine. This corresponded to a rise in the serum Na which went up from 320 to 328 mg./100 c.c. when Miss H. was 'normal', and from 328 to 348 when she was dehydrated. (4) A fall in the concentration of K in the urine, but a small one relative to the increase in the minute volume. It was not observed when the other subject was fully hydrated. Since the rise in the output of water never led to the expected fall in the concentration of K in the urine, and once to no fall at all, it is evident that the excretion of K was being regulated by several factors (see later). (5) A fall in the osmotic U/P ratio. This was not expected, but it seems to be a general finding in this type of diuresis if the output of water is initially low.

(b) KCl. M.T. took 12 g. of KCl by mouth in 100 c.c. of water, once when he was normally hydrated and once when he had lost about 4 % of his body weight by dehydration. His results are given in Table 2. It will be seen that M.T. reacted to the KCl in very much the same way on both occasions, and the changes in his serum K were also identical, the fasting values being 15·2 and 15·4 mg./100 c.c. and the figures 1½ hr. later 20·2 and 20·4 mg./100 c.c. The only finding which needs individual comment is the excretion of Na, which was evidently accelerated on both days by taking the K. It is this

TABLE 2. The effect of KCl on the secretion of urine during dehydration

Time	Urine volume c.c./min.		Urine K m.eq./l.		Urine Na m.eq./l.		Urine urea m.mol./l.		U/P osmotic ratio (see Table 1)	
	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated
Before KCl	0·80	0·54	69	97	115	149	372	357	2·45	2·80
After KCl 1	4·20	3·30	110	121	167	145	90	97	2·05	2·05
After KCl 2	2·82	1·80	131	205	148	124	150	155	2·25	2·60

interrelationship between the excretions of Na and K which prevented a greater fall in the concentration of K in Miss H.'s urine during her salt diuresis and made M.T. when normally hydrated secrete a higher instead of a lower concentration of Na in his urine (Table 2). As in Table 1 the fall in the osmotic U/P ratio will be noted.

(c) *Urea.* 35 g. of urea dissolved in 350 c.c. of water were slowly injected intravenously into Miss T. The time taken over this operation was about 40 min. Table 3 shows the effect. It will be seen that: (1) There was a rise

TABLE 3. The effect of giving 35 g. of urea in 350 c.c. of water intravenously

Time	Serum urea m.mol./l.		Urine volume c.c./min.		Urine urea m.mol./l.		Urine Na m.eq./l.		U/P osmotic ratio (see Table 1)	
	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated
Before injection	4·4	5·0	0·48	0·29	357	487	120	125	2·55	3·05
After injection 1	18·7	21·3	2·05	0·97	533	742	54	15	2·25	2·55
After injection 2	17·3	19·3	2·41	0·88	450	792	99	17	2·28	2·71

in the output of water on both occasions, but a smaller one during dehydration. (2) There was a similar rise in the serum urea on the two occasions. This was accompanied by an increase in the concentration of urea in the urine. (3) There was a fall in the concentration of Na in the urine. The increase in the urine volume was enough to explain this when Miss T. was normally hydrated but not when she was dehydrated, and this fall in the output of Na was accompanied by an equally great fall in the output of Cl. The serum Na did not change, but the serum Cl fell from 391 to 359 mg./100 c.c. when Miss T. was dehydrated, and these changes are probably examples of the ability of urea to produce an alkalosis, a phenomenon which was at one time briefly reported by Adolph [1925]. The fall in the serum Cl was probably the cause of the diminished excretion of Cl but the Na results are not so easily explained. (4) The U/P osmotic ratio fell on both occasions.

A diuresis due to a hypertonic solution might have been expected to take place in a dehydrated person without a fall in the osmotic pressure of the urine or in the U/P osmotic ratio, but the present experiments have all shown that under such conditions the U/P osmotic ratios do fall. It is difficult to explain this observation because it is not known what controls the output

of water when the kidney is secreting a urine with the highest specific gravity of which it is capable. It may be the total osmotic pressure of the urine; it may be the concentration of some specific ingredient. Chaussin [1920], Adolph [1923] and others [Baird & Haldane, 1922; Davies, Haldane & Peskett, 1922] have all considered this problem, and it will be mentioned again later. Be that as it may, the present results are in complete agreement with those of Adolph [1923] and show that dehydrated and normal persons react in essentially the same way to these osmotic violations of their internal environment. The kidney commences at once to excrete the NaCl, KCl or urea, but because of its well-known osmotic limitations it is obvious that it would be forced to draw upon water already present in the body in order to do so completely. Thus the administration of much of a solution more concentrated than the kidney can excrete will inevitably make a person more dehydrated. The salt may be excreted, in which case water already in the body will be excreted with it. If, however, the salt is retained, the osmotic pressure of the body, already higher than normal, will be raised still more. The difference between the osmotic pressure of the administered solution, however, and that of concentrated human urine is no direct indication of the harm which its ingestion is likely to produce.

In one respect the experiments with NaCl and KCl make an interesting contrast with the one in which urea was used. No new principles are involved, but it will be noted that in the two former there was a considerable rise in the concentration of Na and K in the urine, but the changes in the plasma were relatively small. Consequently, the U/P ratios of the ingested ion rose. In the urea experiment there was a large rise in the concentration in the plasma, and consequently, although the urinary concentrations also rose, the U/P ratio fell—and fell, moreover, to less than half its original value.

24 hr. volumes and total solids

The volumes of urine passed daily by these subjects during dehydration varied from 335 to 864 c.o., i.e. from 0·23 to 0·59 c.c./min., but only on one occasion did the minute volume exceed 0·48 c.c. Chesley [1938] found volumes as low as 0·156 c.c./min. following complete abstinence from water for 30–60 hr. The lowest volume passed by Nadal, Pedersen & Maddock's [1941] fasting subject in 24 hr. was 409 c.c., but one of Coller & Maddock's [1935] volunteers secreted only 273 c.c. in 24 hr. when he was taking a dry maintenance diet. Table 4 shows the daily urine output of two subjects who were carrying out balance experiments [Black et al. 1944]. It will be noted that the output of water fell on the first day of the dehydration period and then remained very constant. On this first day the sum of the urea and mineral substances excreted was less than the quantity passed when each person was hydrated. This was largely due to a fall in the output of urea.

TABLE 4. The effect of dehydration upon the specific gravity of the urine
and upon the excretion of urea, minerals and total solids

Day	Urine volume c.c./day	Specific gravity	Weighed solids		Urea and minerals* g./day	Urea g./day
			g./100 c.c.	g./day		
			Subject D.B.			
Prelim. average	1931	1012	2 0	39	34	20
Dehydr. 1	666	1030	5·3	35	29	15
2	652	1033	7·2	47	36	21
3	615	1037	8·7	55	38	24
Rehydr. 1	597	1033	7·6	45	39	27
2	1195	1015	3·7	44	31	26
3	1886	1012	2·2	42	38	22
			Subject J.H.			
Prelim. average	1935	1012	1·6	31	26	15
Dehydr. 1	570	1035	5·3	30	22	13
2	590	1034	5·4	32	30	19
3	595	1034	5·5	33	30	20
4	605	1035	6·3	38	32	21
Rehydr. 1	506	1036	7·1	36	26	20
2	1746	1010	—	—	31	23
3	1990	1010	1·8	36	30	18

* $Na + K + Ca + Mg + Cl + PO_4 + SO_4$.

On the second and subsequent days of dehydration, however, both men excreted more urea, and these findings confirm those of Coller & Maddock [1935]. There was little change in the daily excretion of mineral substances during these dehydration periods [Black *et al.* 1944]. In D.B. the output of 'weighed solids' increased considerably more than that of urea and minerals as dehydration progressed, and it remained high on the rehydration days. The increase in the undetermined fraction of the weighed solids accounted for the rise in the specific gravity of the urine on the second and third days of D.B.'s dehydration. Price, Miller & Hayman [1940] have shown that the proportion of the specific gravity accounted for by measuring the urea, creatinine and all the inorganic salts varies from 70 to 90 % according to the amount of protein in the food. Although the same diet was given to the present subjects throughout the experiments, there was evidence that their endogenous metabolism altered during the period of dehydration [Black *et al.* 1944], and this may have been the cause of the increased excretion of undetermined solids.

Effects produced by controlled rehydration

Table 5 shows the effects of controlled rehydration upon the volume of the urine and upon two of its constituents. All these subjects were on standardized diets before, during and after the days under discussion. The nature and object of the experiments on G.L., O.S. and P.B. will be described later. It will be noted that a huge increase in the consumption of water invariably led to a fall in the output of water. This observation, which seems very remarkable

of water when the kidney is secreting a urine with the highest specific gravity of which it is capable. It may be the total osmotic pressure of the urine; it may be the concentration of some specific ingredient. Chaussin [1920], Adolph [1923] and others [Baird & Haldane, 1922; Davies, Haldane & Peskett, 1922] have all considered this problem, and it will be mentioned again later. Be that as it may, the present results are in complete agreement with those of Adolph [1923] and show that dehydrated and normal persons react in essentially the same way to these osmotic violations of their internal environment. The kidney commences at once to excrete the NaCl, KCl or urea, but because of its well-known osmotic limitations it is obvious that it would be forced to draw upon water already present in the body in order to do so completely. Thus the administration of much of a solution more concentrated than the kidney can excrete will inevitably make a person more dehydrated. The salt may be excreted, in which case water already in the body will be excreted with it. If, however, the salt is retained, the osmotic pressure of the body, already higher than normal, will be raised still more. The difference between the osmotic pressure of the administered solution, however, and that of concentrated human urine is no direct indication of the harm which its ingestion is likely to produce.

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Prelim. average	1935	1012	1·6	31	26	15
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2	590	1034	5·4	32	30	19
3	595	1034	5·5	33	30	20
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* $Na + K + Ca + Mg + Cl + PO_4 + SO_4$.

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TABLE 5. The effect of controlled rehydration upon the volume of the urine and the percentage of urea and of chlorides in it

Subject		Intake of water + water of metabolism c.c./24 hr.	Urine volume c.c./24 hr.	Urea mg./100 c.c.	Cl mg./100 c.c.
D.B.	D*	680	615	3980	777
	A*	5230	597	4600	434
J.H. ·	D	474	605	3450	930
	A	3804	506	3900	605
G.L. (High salt intake)	D	400	635	2580	1230
	A	3100	510	3140	1000
G.L. (Low salt intake)	D	400	425	4170	870
	A	3120	420	4370	318
O.S.	D	400	690	1840	1320
	A	2950	610	2590	945
P.B.	D	400	720	2530	1315
	A	4390	607	3730	580

* D = during the last 24 hr. of dehydration; A = during the first 24 hr. after rehydration began

·at first sight, was made by Coller & Maddock [1935], but they do not appea to have realized its significance. The effect, which is always coupled with fall in the concentration of urinary Cl and a rise in the concentration of urea can be produced most easily when the diet contains 9 g. or more of salt a day but G.L. showed it even when there was a much smaller quantity in his food In all these subjects the concentration of urea in the urine was higher on th first day of rehydration than on any preceding one. The output of urea some times rose and sometimes fell according to the magnitude of the fall in th volume of the urine and the rise in the percentage of urea in it. As soon a rehydration was completed the urine volumes became as great as they ha been during the preliminary period.

Salt intakes and urine volumes

It will be seen in Table 4 that when D.B. and J.H. were dehydrated the were both passing comparatively large volumes of urine, and it was felt tha the main reason for their size might be the amount of salt and nitrogenou matter which the subjects had consumed. To test this the following experi ment was arranged. Three subjects were placed upon a standardized dry die which contained little salt. For the first 3 days G.L. and P.B. took 10 g. o NaCl additional to their food and all drank as much water as they wished On days 4, 5 and 6 all the subjects abstained from water but maintained thei intakes of food and salt. On day 7 all the subjects rehydrated themselves On day 8 G.L. and P.B. gave up taking the added NaCl and O.S. began t do so, and on this and the following day a free intake of water was encouraged On day 10 the second dehydration period began and lasted till the end o day 12. The last day was again devoted to studying rehydration effects. Th

results of this experiment are shown in Table 6, and the influence of the salt intake on the urine volume is clearly demonstrated by the first two columns. The same effect is shown by G.L.'s results in Table 5.

TABLE 6. The effect of the intake of salt upon the volume, specific gravity, osmotic pressure and the urea-mineral relationships of the urine

	Average urine volume c.c./day		Average specific gravity		Average conc. of Cl m.eq./l.		Average conc. of urea m.mol./l.		Average conc. of urea ÷ 2 (Na ÷ K) m.osmol./l.	
Subject	Low salt	High salt	Low salt	High salt	Low salt	High salt	Low salt	High salt	Low salt	High salt
G.L.	413	700	1035	1031	163	332	620	356	1049	1036
O.S.	371	815	1033	1033	118	344	507	227	890	993
P.B.	365	633	1035	1034	164	339	564	386	1080	1160

The urea clearances

Table 7 shows some data about the excretion of urea by D.B. and J.H. Similar figures could have been given for the other subjects. It will be noted that with the onset of dehydration the clearances (UV/B) fell, and that as

TABLE 7. The effect of dehydration and partial rehydration upon the treatment of urea by the kidney

Subject	Day of exp.	Urine volume c.c./min.	Blood urea mg./100 c.c.	Urine urea mg./100 c.c.	U/P urea ratio	Urea clearance c.c./min.	Urine chloride m.eq./l.
D.B.	Prelim. av.	1·34	33	1040	32	42·0	86
	Dehydr. 1	0·46	40	2210	55	26·0	217
	2	0·45	54	3230	60	27·0	202
	3	0·43	58	3980	69	29·5	219
	Rehydr. 1	0·41	50	4600	92	38·0	122
J.H.	Prelim.	1·34	20	780	38	52·0	77
	Dehydr. 1	0·40	28	2250	81	31·8	268
	2	0·41	42	3200	77	31·4	246
	3	0·41	44	3400	77	31·8	244
	4	0·42	47	3450	74	30·2	263
	Rehydr. 1	0·35	45	3900	87	30·5	170

dehydration progressed the blood ureas rose but the clearances did not change again. Urea clearances are well known to vary with the urine volumes when these are less than 2·0 c.c./min. Incidentally, this is equally true for dehydrated as for normal persons, for when Miss H., M.T. and other dehydrated subjects took large doses of hypertonic salt (Tables 1 and 2), the resulting diuresis always raised the urea clearances in the characteristic fashion. It was, therefore, the fall in the output of water with the onset of dehydration which reduced the urea clearances of D.B. and J.H. and hence (see Table 4) the total amount of urea excreted. The urea which was retained accumulated in the body fluids, and raised the concentration in the blood. This in turn allowed the output to rise without any change taking place in the clearances. Some part of the rise in the blood urea during dehydration was due to the

increased production of urea which was taking place at that time [Black *et al.* 1944], and it was presumably this extra production which forced the outputs to rise above their pre-dehydration levels. It will be appreciated from Table 7 that throughout the 3 or 4 days of dehydration the percentage of urea in the urine rose *pari passu* with that in the blood. Had it not done so the U/P urea ratio and the clearances would have fallen. There is no evidence that either D.B. or J.H. ever produced the highest concentration of urinary urea of which they were capable, and it must remain uncertain how much further they could have raised the percentage of urea in their urines under the conditions to which they were subjected at that time. The administration of urea to Miss T. when she was dehydrated, however, probably raised the concentration of urea in her urine to its maximum, for in spite of a rise in urine volume her urea clearances remained low until the blood urea began to fall.

The most interesting figures in Table 7 are those for the first day of rehydration. In spite of a fall in urine volume D.B.'s clearance rose and J.H.'s did not fall. The percentage of urea in the plasma fell and that in the urine rose, and both these changes increased the U/P ratio. These increases, coupled with a fall in the minute volume of the urine, are a direct refutation of Chesley's [1938] contention that when the urine flows are reduced below a certain 'critical' limit, the urea clearances and the urine volumes necessarily vary together.

A general explanation of these findings will now be given, but there are many difficult points still to be cleared up. (a) The volume of the urine varied with the intake of salt (Table 6), or, more precisely, with the amount entering the distal part of the nephron [Shannon, 1942 *a, b*], where there seems to be a limiting concentration for chloride [Adolph, 1921; Baird & Haldane, 1922] and for other osmotically active substances [Davies, Haldane & Peskett, 1922]. Table 7 shows that in D.B. and J.H. the urinary Cl reached its ceiling on the first day of dehydration. These concentrations were not so high as the 'maximum' set up by Miss H. or by Davies *et al.* [1922] subject after large doses of NaCl by mouth; neither were they so high as the concentration produced by O.S., G.L. and P.B. on their high salt diets. J.H., moreover, one morning some time after this experiment took 20 g. of NaCl in 400 c.c. of water and subsequently excreted 1·14% of Cl in his urine (322 m.eq./l.). It may be assumed therefore that both men could have produced higher concentrations of Cl in the urine given the appropriate conditions. Nevertheless, the effects of rehydration show that the amount of salt to be excreted must have been one of the agents fixing and maintaining the output of water during the days of dehydration. With the onset of rehydration the concentration of chloride in the plasma fell and presumably less reached the distal part of the nephron. Possibly also there was some change in the hormone balance. At all events, so much less Cl presented itself for excretion that the output of

water fell as did the percentage of Cl in that water (Table 7). This last enabled the kidney to raise the concentration of urea [Chaussin, 1920]. (b) Shannon [1936] showed that the glomerular filtration rates (measured by the creatinine clearances) and the urea clearances of dogs tended to increase with rehydration even before the urine volumes began to rise. He found too that the urea/creatinine clearance ratios were higher during rehydration than in dehydration. If the kidneys of our subjects were responding to rehydration in the same way, one or both of these changes may have accounted for the rise in D.B.'s urea clearance on the first day of rehydration. In keeping with this is the fact that D.B.'s glomerular filtration rate did fall slightly during dehydration and presumably therefore may have returned to normal when rehydration began.

The excretion of the minerals

It was shown in the balance experiments [Black *et al.* 1944], and in Table 4, that the output of Na, K and Cl and of total minerals changed little during dehydration. Table 8 now shows that D.B.'s output of Na per litre of urine

TABLE 8. The relationship of urine volume, and plasma levels to the excretion of Na, K and Cl

	Plasma			Urine			
Day of exp.	Na mg./ 100 c.c.	K mg./ 100 c.c.	Cl mg./ 100 c.c.	Volume c.c./day	Na mg./ 100 c.c.	K mg./ 100 c.c.	Cl mg./ 100 c.c.
Prelim. average	340	19·3	377	1931	184	107	301
Dehydr. 1	346	19·0	388	666	370	470	769
3	369	15·1	406	615	483	520	777
Rehydr. 1	354	16·0	380	597	332	336	434
2	340	16·6	374	1886	137	129	255

as higher on the third day of dehydration than it had been on the first. his may reflect the rise in the serum Na, but the concentration of K was so higher in spite of a fall in the serum K. Furthermore, on the first day of hydration the percentage of K in the urine fell greatly although the serum K as rising, and the urinary concentration of Na was lower than it had been a the first day of dehydration, although the serum Na was still above its ormal level at that time. It is obvious, therefore, that the excretion of these inerals was not regulated, as was that of urea, simply by their concentrations in the plasma. Recent work, reviewed by Shannon [1942b], has indicated hat so far as NaCl is concerned the suprarenal and gonadal hormones, and he anti-diuretic hormone of the pituitary, may all be involved.

The relationship between the excretion of osmotically active
substances and the urine volume

It has generally been assumed [Smith, 1937] that the extent to which the urine can be concentrated is limited by the total osmotic pressure which it exerts. A more complicated but possibly more correct view may be that the limiting factor is the sum of the U/P ratios of all the urinary constituents. The reciprocity between Cl and urea, which was so assiduously studied by Chaussin [1920], can most easily be explained along such lines. The effect of controlled rehydration, moreover (Tables 4, 5 and 7), and the effects of salt (Table 6), can be accounted for best in that way. There is some evidence, however, which suggests that it is more often the specific effect of one of the urinary constituents which fixes the volume [Adolph, 1923]. The concentration of urea in human urine, for instance, seldom exceeds 4%, and it is well known [Davies *et al.* 1922] that there is a ceiling for Cl, or for Cl and HCO_3, and that a diuresis results if the kidney is called upon to excrete more of these substances than it can accommodate in the volume of urine suggested by the water requirements of the body at that time. These two mechanisms are not incompatible and, given the appropriate conditions, either could probably be reproduced in the same person. In other words, both operate. According to some authors, however [Baird & Haldane, 1922; Davies *et al.* 1922; Gilman & Kidd, 1938], the maximum concentrations of Cl and of urea may coexist. The present experiments suggest that they can only do so under very special conditions. The experiment, which is illustrated in Table 6, showed that the addition of 10 g. of NaCl to the diet increased the urine volumes by about 300 c.c. · (Incidentally this volume was insufficient to accommodate 10 g. of NaCl even at the maximum concentration of about 2% (342 m.eq./l.). Yet fully 10 g. of NaCl were excreted when the intake of salt was raised, and considerably more than had been excreted when the intake of salt was low. The two facts which integrate these observations are: (1) when the subjects were on a low salt intake the concentration of Na in the urine was far below the maximum; (2) when the intake of salt was high the total volume of the urine was always more than enough to accommodate 10 g. of NaCl at the accepted maximum concentrations.) It is evident, therefore, that the NaCl to be excreted was one of the factors fixing the urine volumes when the intake of salt was high, and, since at that time the urinary concentrations were round about the highest which the body can produce, the Cl to be excreted may have been the only factor controlling the output of water. The present experiments do not point to any one substance as being solely responsible for fixing the urine volumes when the intake of salt was lower, but the effects of rehydration on G.L. at that time, and upon D.B. and J.H., suggest that NaCl must have been playing some part. These subjects all showed the urinary

changes characteristic of rehydration in controlled stages, although none of them had had maximum concentrations of Cl in their urines during dehydration (see Table 7). Furthermore, the specific gravity of the urines, and the average osmotic pressure exerted by the urea together with the Na and K salts, were about the same whether the intakes of salt were high or low (see Table 6). The contributions of urea and of the minerals, however, varied very much, and hence it seems probable that the total osmotic pressure for these substances had the final say in the volume of water to be excreted during dehydration. It is possible that, by raising the blood ureas, 'maximum' concentrations of urea could be produced in the urine, and that this could be done without reducing the concentration of minerals, but the present experiments give no indication that during dehydration 'maximum' concentrations of urea and of minerals should be expected simultaneously. Further work on these matters is in progress.

PRACTICAL CONSIDERATIONS

Leaving aside the difficult points of detail which often tend to obscure the general principles, the present experiments clearly show that raising the salt intake of a person who is kept short of water makes his dehydration worse by increasing the volume of his urine. Diet during dehydration, therefore, should be as free of salt as possible and, logically, everything should be done to diminish the urinary solids calling for excretion. Whether sea water should be taken by a shipwrecked mariner demands a little more thought, for this involves the consumption of water as well as salt. If the subjects G.L., O.S. and P.B. had taken their 10 g. of salt in the form of sea water (or 3.3% NaCl), and if we were to consider only the increased output of water and salt and the volume of sea water drunk, we might reach the interesting if somewhat dangerous conclusion that the men would have been none the worse for their potations. Further reflexion, however, suggests that they would have been worse, for their plasma sodiums, and, consequently, in all probability the osmotic pressure of their body fluids, would have been higher, and, had they taken more than 10 g. of NaCl, or had their basal diets not had very little salt in them, they would certainly have been worse, for the concentration of Cl in their urines had already reached its limits, and consequently the next 300 c.c. of sea water would have had a much more disastrous effect.

The results of the present experiments, and a general consideration of the known facts about the specific gravity and other characteristics of the urine passed during a period of water deprivation, suggest that a dehydrated man can do himself no good by drinking his own urine. By so doing he is merely asking his kidneys to repeat work which they have already done, and cannot be expected to do better.

SUMMARY

1. In human dehydration produced by 3 or 4 days without water, th glomerular filtration rates and the diodone clearances fell little if at all i men who had lost up to 7 % of their body weights.

2. Large doses of NaCl, KCl and urea led to diuresis even during dehydra tion. The increased flow of urine was accompanied by a fall in the urine/plasm osmotic ratio, and each diuretic produced specific changes in the compositio of the plasma and the urine.

3. When the diet was standardized the daily output of water depende upon the intake of salt. When this was moderately high (5–15 g./day), th output of water could always be demonstrated to fall with the onset of r hydration, and the urine at this time contained more urea and less NaCl p litre than it had done during the days of dehydration.

4. Dehydration reduced the urea clearances, mainly if not solely by r ducing the urine volumes. In the early stages of rehydration the clearance did not fall further—and in at least one case they rose—in spite of the d creased output of water characteristic of that period.

5. The output of minerals during dehydration and rehydration was regu lated by factors other than their concentrations in the plasma.

6. When the intake of NaCl was sufficiently high the output of water ma have been regulated solely by the salt presenting itself for excretion, but th was certainly not so when the intake of salt was low. It is suggested that th total osmotic pressure of the urine was responsible at all times.

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THE RENAL FUNCTION OF NEWBORN INFANTS

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In a recent paper McCance & Young [1941] have shown that normal infants aged 7–14 days excrete a urine which is persistently more dilute than that of adults. They have found that in premature and full-term infants the urea and mineral clearances are always low by adult standards [Young, Hallum & McCance, 1941] and that almost all the electrolytes filtered off in the glomeruli of many infants must be re-absorbed by the tubules. This may be one of the reasons why infants are unable to increase the concentration of urinary solutes in the way adults do when the output of urine falls. However, a detailed analysis of the process of elaboration of urine at low minute volumes in newborn children would seem to suggest that the low mineral clearances of the infantile kidney are unlikely to be the only factor which operates for the excretion of a persistently dilute urine. McCance & Young's work supplies evidence for the view that the proximal tubule of the infantile kidney is capable of reabsorbing 'threshold' substances at a rate which, favoured by the low filtration rate characteristic for the glomerulus of the newborn infant, is at least equal to the reabsorptive capacity of the adult tubule. It seems therefore permissible to assume that, just as in the adult kidney, a blood isotonic [Walker, Bott, Oliver & MacDowell, 1941] or more likely a blood hypotonic [Shannon, 1942a] fluid is formed in the proximal renal tubule of the infant. A fluid of such a composition and flowing at such low velocity would invariably be highly concentrated when passing through the distal tubules of a normal adult kidney. The excretion by the kidney of the newborn of a dilute urine at low minute volumes suggests, therefore, that the distal tubules of the newborn infant function in a manner different from that of the adult.

The possibility that several factors may be involved in the process of water reabsorption by the distal tubule has recently been postulated [Shannon, 1942a]. However, there is so far no evidence against regarding the posterior pituitary antidiuretic principle as the major determinant of the final concentration of the urine at low minute volumes. The question arises therefore whether the persistently high dilution of the urine of newborn infants might be due to some inadequacy of the posterior pituitary-renal mechanism. It was realized that such an inadequacy might be connected with one or several of the following possibilities: (1) Lack of response of the infantile kidney cells to the

circulating antidiuretic hormone. (The question of the developmental state of the human tubules at birth, as discussed by Gersh [1937], Gruenwald & Popper [1940] and McCance & Young [1941] should here be considered.) (2) Insufficient production of the antidiuretic factor by the posterior pituitary lobe of the newborn infant. (3) Insufficient development of the mechanism which integrates the production and liberation of the antidiuretic hormone with the functional state of the tubular epithelium. The aim of the present paper was an attempt to investigate the first of these possibilities. An investigation of the urinary concentration of infants during the first 6 days of extra-uterine life and its relation to fluid intake were subsidiary subjects.

METHODS

Specimens of urine were obtained from twenty-three normal, full-term infants aged 8–135 hr. Male children only were investigated. The urine samples were collected in small test-tubes attached by elastic strapping. The movements of the children were not restrained. Twelve healthy men aged from 21 to 37 years served as adult controls. Urine samples were put on ice immediately after collection.

Urinary freezing-point depressions (Δ) were estimated with a Beckmann apparatus for small quantities of fluid. Δ values were converted into milli-osmolar concentration by the usual formula: C (in milli-osM.) $= \Delta/1.86 \times 1000$. Corrections for the sediment which forms on cooling were obtained by the following procedure: the volume of the urine sample was measured at room temperature, the sample cooled to $-0.5°$ C., centrifuged, the supernatant fluid decanted, the sediment dissolved in twice the original volume of distilled water and the freezing-point depression of this solution determined. The figure so obtained was multiplied by two and added to the Δ of the supernatant fluid.

Heller's [1941] apparatus was used for the determinations of specific gravity. The pituitary (posterior lobe) preparation employed was B.D.H. posterior pituitary extract.

RESULTS

The effect of posterior pituitary extract on the urinary secretion of newborn infants

An assessment of the action of posterior pituitary extract on the kidney of the newborn infant meets considerable technical difficulties. It is customary to estimate the response of the mammalian kidney to the antidiuretic hormone by the inhibitory effect of a given dose of posterior pituitary extract on a water diuresis. The inhibition in turn is measured by the decrease of urine flow in the unit time. This technique is hardly applicable to the newborn infant because (1) it is difficult to be sure that none of the urine voided has been lost, and (2) because newborn infants cannot be made to empty their bladder at regular

intervals. (It was felt that the use of a catheter was not permissible in an investigation on healthy infants.) The following method was therefore used
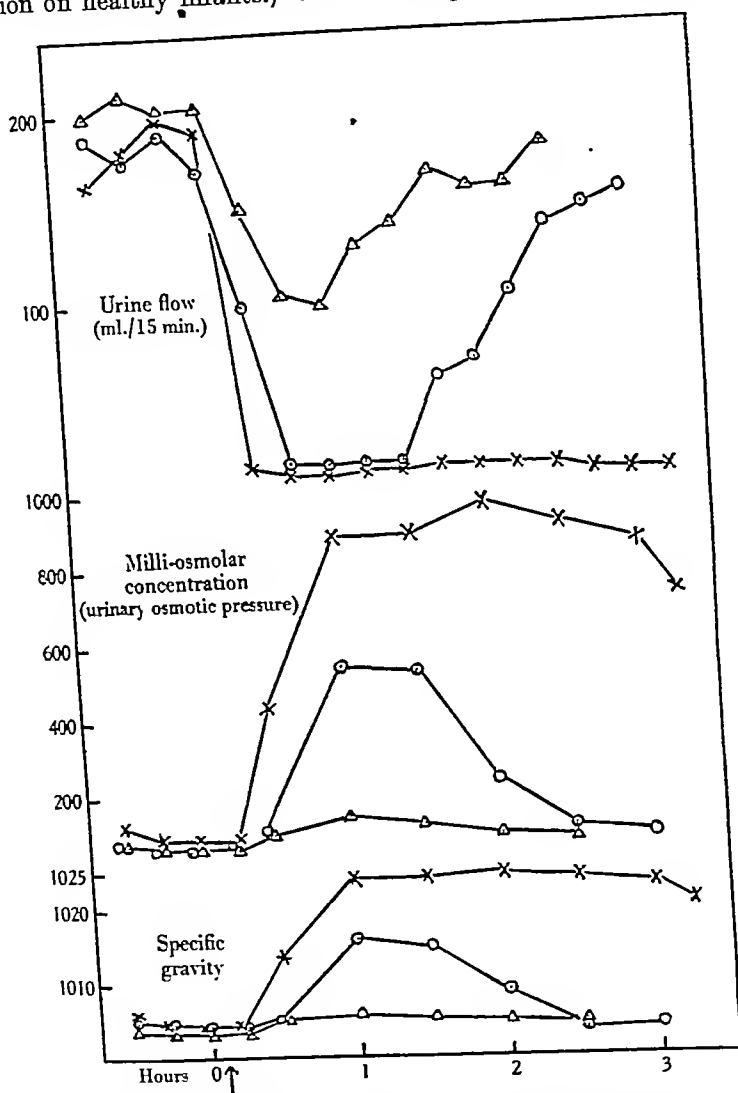

Fig. 1. The effect of intramuscular injections of posterior pituitary extract on urine flow, urinary osmotic pressure and urinary specific gravity of a normal adult subject. (H. ♂, 36, body surface 1·90 sq.m.) △——△ 12·5 mU. sq.m., ⊙——⊙ 25 mU. sq.m., ×——× 150 mU. sq.m. Injections at the time marked by arrow. The subject drank 100 mL. water per sq.m. body surface at intervals of 15 min. throughout the duration of the experiment.

to obtain evidence as to whether injections of posterior pituitary extract had an effect on the urinary secretion of the newborn child: Changes of urinary osmolar concentration (calculated from Δ) rather than changes of urinary

volume were used as criterion of the effect of the antidiuretic principle. Fig. 1 shows that this method of gauging the effect of an injection of posterior pituitary extract gives as faithful a picture of the action of the antidiuretic factor on the urinary secretion of the adult as measurements of changes of the urine volume. The possibility of a dissociation by the infantile kidney of the effect of the antidiuretic factor on the urinary volume and the urinary concentration cannot at present be discounted. However, such a dissociation has, to the author's knowledge, hitherto not been observed in normal subjects.

Estimations of concentration rather than volume obviate the necessity of quantitative collections of urine. The elimination of the second difficulty, viz. the collection of urine specimens of newborn infants at specified times, was attempted by the use of the following procedure: (1) A urine specimen from a newborn child was collected and a given dose of posterior pituitary extract injected intramuscularly. (2) Samples of urine voided spontaneously at the two following occasions were then taken and the Δ of all specimens determined. (3) In order to compare the effect of the antidiuretic principle on the urinary secretion of the newborn child with that on the adult, an equivalent amount of posterior pituitary extract was injected into adults who drank 100 ml. water per sq.m. body surface at intervals of 15 min. throughout the duration of the experiment. (4) Urine samples of adults were collected at exactly the same times as those at which the infants had emptied their bladders spontaneously.

Fig. 2 illustrates such experiments. It is clear that the analysis of urine samples collected at such irregular times and at such comparatively long intervals cannot give an accurate picture of the effect of the posterior pituitary extract. However, it will be noticed that in the case of the newborn infant the first urine collected after the injection was somewhat more concentrated than the urine obtained before the injection was made. The second sample after the injection shows a return to the pre-injection level. This slight rise of urinary concentration and the subsequent return to the normal occurred regularly in experiments of this type and is unlikely to have been accidental ($t = 3.06$, $P < 0.05 > 0.02$). It may therefore be taken as indicating that posterior pituitary extract may have some slight effect on the kidney of the newborn, though a comparison of this effect with the effect of an equivalent dose on the urinary concentration of an adult subject (Fig. 2) suggests a high degree of insensitivity of the renal tubules of the newborn child to the antidiuretic hormone.

It will be noted that the concentration of the urine samples taken immediately before the injection of the posterior pituitary extract are much the same for newborn infants and adults. It was to achieve this that the adult controls drank large quantities of water before an injection of posterior pituitary extract was made. The subsequent doses of 100 ml. water per

sq.m. body surface taken every 15 min. were sufficient to maintain their urinary concentration at a level comparable to that of infants aged over 4 days (Fig. 3). It will be shown later that children aged 1–4 days were usually found
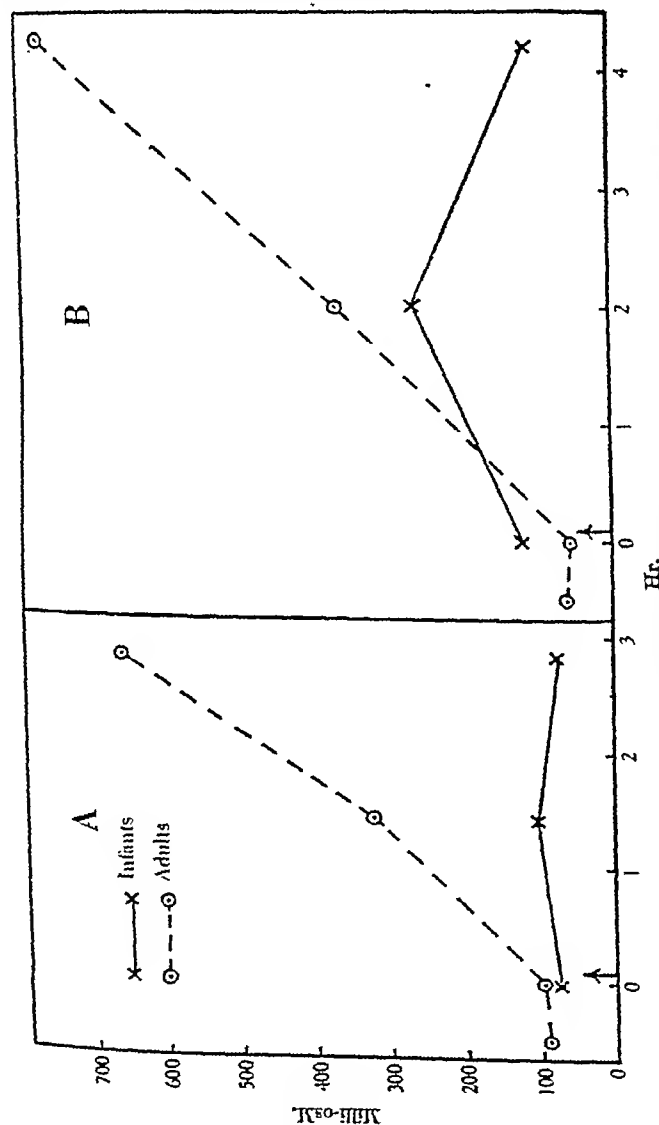

Fig. 2. A comparison of the effect of equivalent doses of posterior pituitary extract on the urinary osmotic pressure (in terms of milli-osmolar equivalents) of newborn infants and of adults. (A) At the time marked by arrow intramuscular injection of 125 mU. sq.m. into the infant Sh. (5th day of extra-uterine life) and the adult subject Pa. Absolute doses: 25 mU. and 210 mU. respectively. (B) At the time marked by arrow intramuscular injection of 250 mU. sq.m. into the infant Ma. (5th day of life) and the adult subject Du. Absolute doses: 50 mU. and 500 mU. respectively.

to excrete a considerably more concentrated urine. Two such children (baby Cap. aged 84 hr. and baby Car. aged 90·5 hr.) were injected with 150 mU. posterior pituitary extract per sq.m. Urine samples collected before the injection gave a concentration of 664 and 462 milli-osM. respectively. The first sample after the injection (obtained after 50 min. in the case of baby Cap.

and after 86 min. in that of baby Car.) yielded values of 643 and of 441 milli-osM. respectively. It will be seen that the slight increase of urinary concentration after the injection of posterior pituitary extract which was regularly noticed in infants who secreted a strongly hypotonic urine (Fig. 2) was not observed in these cases.

The means by which 'equivalent' doses for newborn infants and for adults had been established have still to be discussed. The doses used (Fig. 2) were based on the relative areas of body surface, since it appears that rates of urinary output at all ages are correlated most accurately with the body surface [Adolph, 1933]. Following McCance & Young [1941] the average ratio of adult to infantile surface area has been taken as 8·25, and the doses of posterior pituitary extract injected were calculated accordingly. McCance & Young point out that in the newborn infant body surface area, though proportional to the output of urine, is not proportional to basal metabolic rate and kidney weight. The use of the surface area as a basis for the calculation of doses of posterior pituitary extract is therefore less acceptable for infants than for adults. However, it will be noticed (Fig. 2A) that 125 mU. posterior pituitary extract per sq.m. had been chosen as the standard dose. Several other experiments gave essentially similar results when 250 mU. per sq.m. had been injected (Fig. 2B). This means, as Fig. 1 shows, that the adult controls received 5 or respectively 10 times the minimum effective dose of the antidiuretic hormone. The use of such large doses makes it *a priori* unlikely that the choice of another basis for the calculation of equivalent doses would have influenced the results significantly. It should also be pointed out that the absolute dose injected into infants receiving 250 mU. posterior pituitary extract per sq.m. amounted to as much as 50 mU. This is a dose which, injected into an healthy adult, produces a marked inhibition of a water diuresis (Fig. 1). The choice of the basis of comparison was thus of comparatively little importance.

The osmolar concentration of urines of infants aged 8–135 hr.

McCance & Young [1941] furnished data for the osmolar concentration of urines of normal children aged 7–14 days. The infants investigated in the present series were younger than those of McCance & Young, and the data are therefore complementary to the figures of these authors. Urinary osmotic pressures were assessed by determining freezing-point depressions. Where possible the first urine voided after birth was analysed and the investigation prolonged to the 6th day of life. Children of this age were chosen for two reasons. First, fluid intake during the first week of life is much more variable than during the second. Secondly, clinical disturbances of the water balance are more frequent during the first days of life.

Fig. 3 shows results obtained from a series of twenty-three infants. It will be seen that most infants excreted a comparatively concentrated urine during

the first 2 days after birth. The average concentration decreased rapidly during the 3rd day. Urines voided on the 4th, 5th and 6th days were, with few exceptions, very dilute. McCance & Young's findings suggest that they remain so for at least another week. Facilities to obtain blood samples from the infants investigated in this series were not available. To obtain some information about the serum/urine osmotic pressure ratio it was, therefore, unavoidable to rely on published data for values of the average serum osmotic pressure of
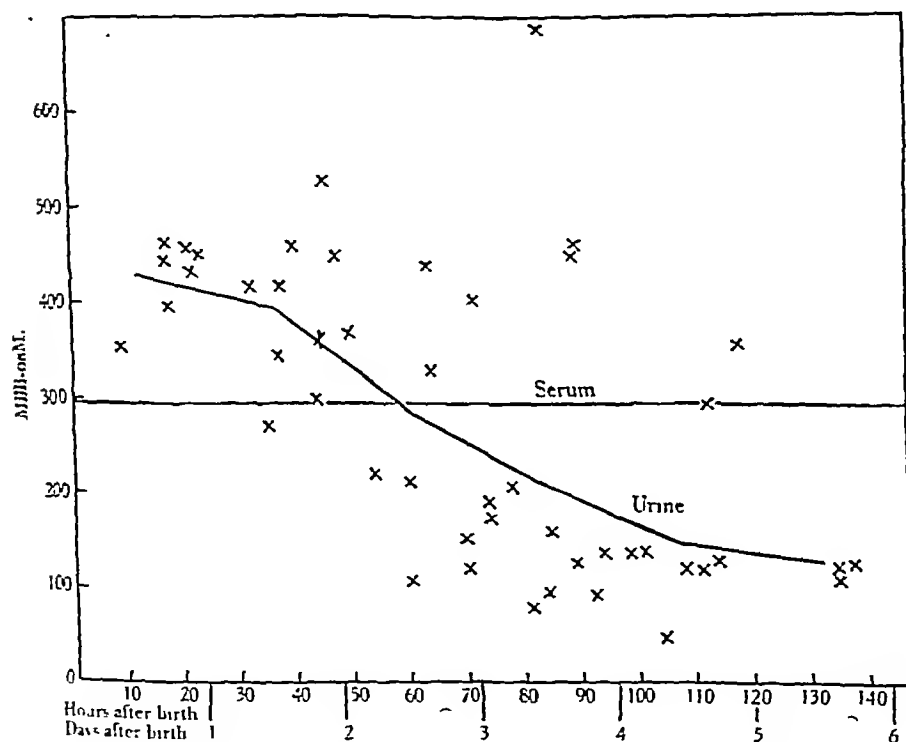

· Fig. 3. Urinary osmotic pressures (in terms of milli-osmolar equivalents) of a series of newborn infants.

· infants. Füth & Wirz [1929] who determined the freezing-point depression of serum samples taken during the first hour after birth found a serum osmotic pressure equivalent to $295·8 \pm 8·72$ milli-osM. (recalculated). This figure would seem to be in agreement with the data of McCance & Young [1941] who, using a different method, obtained a serum osmotic pressure equivalent to approximately $310·9 \pm 14·7$ milli-osM. for infants aged 7–14 days. These figures make it very likely that the infants on which the data of Fig. 3 are based, excreted a slightly hypertonic urine during the first 2 days after birth.

However, it should be emphasized that even these comparatively high concentrations were still much below the average concentrations of urines ex-

creted by a group of normal adults (Fig. 4), and this in spite of the fact that the subjects chosen were having their full complement of fluids (including a minimum of six cups of strong tea per day), whereas the fluid intake of infants during the first and second days of life was much restricted (Fig. 5). The average values of 'day' and 'night' urines of our adult series agree well with Koranyi's [1897] figures. In an investigation on four normal men he found
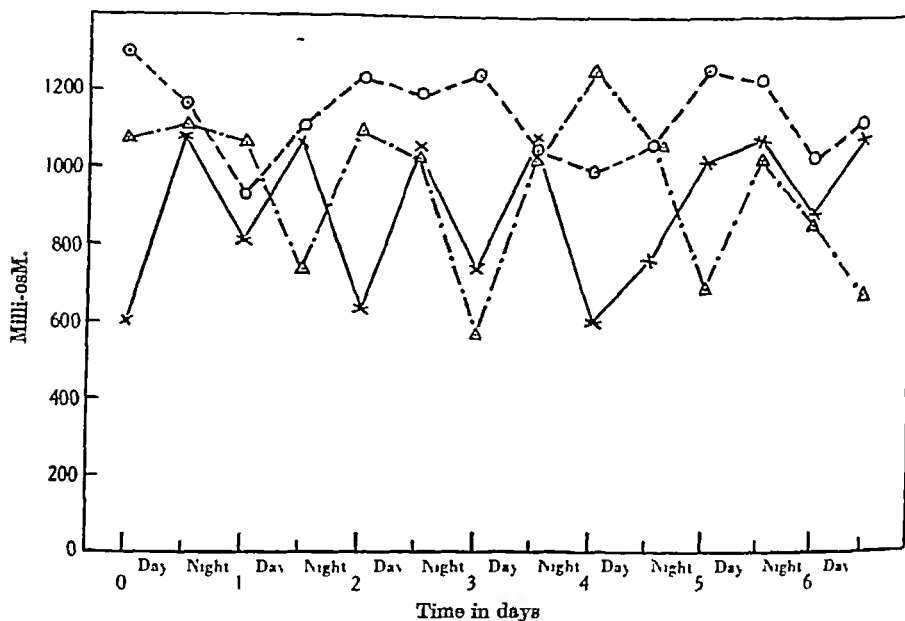

Fig. 4. Urinary osmotic pressure ε(in terms of milli-osmolar equivalents) of three normal adults. ×——× subject H., ⊙---⊙ subject P., △–·–·–△ subject R. 'Night' urines collected between 10 p.m. and 10 a.m., 'day' urines collected between 10 a.m. and 6 p.m. *Note.* The scale is different from that of Fig. 3.

the following milli-osmolar concentrations (recalculated from freezing-point depressions) for urines collected during 4 hr. periods: 7 a.m. to 11 a.m.: $1121 \pm 76 \cdot 6$; 11 a.m. to 3 p.m.: $1205 \pm 108 \cdot 6$; 3 p.m. to 7 p.m.: $1208 \pm 128 \cdot 1$; 7 p.m. to 11 p.m.: $1052 \pm 251 \cdot 8$; 11 p.m. to 3 a.m.: $1226 \pm 136 \cdot 4$; and 3 a.m. to 7 a.m.: $1260 \pm 90 \cdot 9$.

The influence of the daily fluid intake on the urinary concentration of newborn children

A number of factors may be involved in the comparatively sudden decrease of urinary concentration observed in infants during the third day of extra-uterine life (Fig. 3). Metabolic changes resulting from adaptations to extra-uterine life and hormonal changes resulting from the separation from the maternal organism may be considered. Further investigations will be needed

to establish their possible significance in connexion with the metabolism of water and with kidney function. However, one factor possibly concerned with the urinary concentration of newborn infants has, to some extent, been investigated. This factor is the influence of the daily fluid intake. If 24-hourly fluid intakes are plotted against urinary osmotic pressure (in terms of milliosmolar equivalents) it will be seen (Fig. 5) that the urinary concentrations
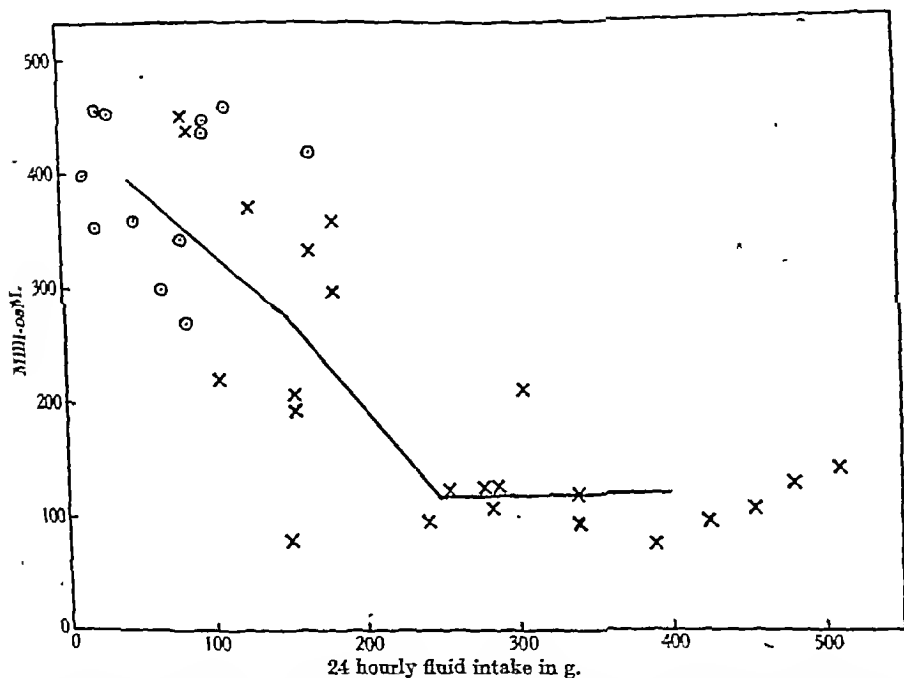

Fig. 5. Relation between fluid intake and urinary osmotic pressure (in terms of milli-osmolar equivalents) of a series of newborn infants. ⊙ infants aged 8–48 hr., × infants aged 49–135 hr.

fall as the daily fluid intake increases. The correlation coefficient for the data represented on Fig. 5 is highly significant ($r = -0.78$, $P < 0.001$), and a relationship between fluid intake and urinary concentration is, therefore, very probable. However, in view of the possible involvement of other, hitherto uninvestigated, factors, it would be rash to assess the degree of causal connexion between the two variants.

DISCUSSION

To what extent do the results of this investigation support the hypothesis that one of the factors responsible for the difference in the function between the kidney of the newborn infant and that of the adult is an inadequacy of the posterior pituitary regulatory mechanism? It has been shown that a dose of posterior pituitary extract which had a pronounced inhibitory effect on the

water diuresis of adults had very little effect on the urinary concentration of newborn children. The question arises whether we are entitled to compare the water diuresis of the adult with the 'normal,' urinary secretion of the newborn infant ingesting adequate amounts of milk at four hourly intervals. The two states are well comparable in so far as the average osmotic pressures of the respective urines are concerned, but they differ in that very low urinary osmotic pressures comparable to those which are maintained for days and very likely weeks by newborn infants, seem to be linked invariably with a relatively much higher minute volume in the adult. To produce such low urinary osmotic pressures in adult healthy mammals a water diuresis has to be instituted. A dissimilarity of the urinary minute volumes can, therefore, hardly be avoided if normal adults should be used for the purpose of comparison.

However, there are conditions in which adult mammals excrete a urine of low concentration at a comparatively low rate. This may occur in cases of abnormal pituitary function such as diabetes insipidus. For example: Shannon [1942a, Table II] mentions a dog with experimental diabetes insipidus which excreted a urine of $\Delta = -0.22°$; the corresponding rate of urine flow was 1·57 ml./min. This may be compared with a normal dog of similar body weight which excreted a urine of similarly low concentration ($\Delta = -0.26°$), but in this case at a rate of 10·00 ml./min. [Shannon, 1942b, Table III]. When the urine flow of the latter animal was decreased to a value comparable to that of the diabetes insipidus dog, i.e. to a rate of 1·52 ml./min. by an infusion of small amounts of pituitrin, the urinary concentration rose to $\Delta = -1.64°$. The similarity in the behaviour of the minute volume/concentration ratio between an organism suffering from diabetes insipidus and the newborn infant suggests the possibility of a posterior pituitary inadequacy in the latter. Are there any other resemblances between the renal function of the newborn child and that of cases of diabetes insipidus? McCance & Young [1941] have shown that the newborn infant's clearances of sodium, chloride and potassium are significantly lower than those of adults. The influence of such factors as the glomerular filtration rate on the clearance value of these threshold substances must not be discounted, but it should be remembered that an increase of the reabsorptive capacity of the proximal tubule for sodium (and chloride) is held to be one of the fundamental derangements in diabetes insipidus [Shannon, 1942 a, b]. It appears thus possible that the low sodium and chloride clearances of newborn infants may partly be due to an insufficient control of the tubular electrolyte reabsorption by the posterior pituitary lobe. Moreover, the findings of Stehle [1927] and others suggest that the renal excretion of potassium as well as that of other ions is influenced by the posterior pituitary hormones.

The foregoing considerations suggest that the nephron of a well-hydrated infant may be pictured as functioning in some such manner as the following:

The insufficiently developed glomerulus delivers its filtrate to the proximal tubule at a low rate [Barnett, 1940; McCance & Young, 1941]. Owing to the low velocity of flow and possibly to the lack of posterior pituitary control, electrolyte reabsorption is higher than in the adult, and a hypotonic fluid reaches the distal site of the action of the antidiuretic hormone. The low velocity of flow in conjunction with the low tonicity of the tubular fluid constitute highly favourable conditions for the action of the antidiuretic hormone, but owing to an insufficiency of the posterior pituitary-renal mechanism little water reabsorption occurs, with the result that at relatively low filtration rates a small volume of a urine of low osmotic pressure is elaborated.

Assuming then that the renal function of newborn infants resembles in some points that of an adult subject suffering from total or partial diabetes insipidus, is there any justification in suggesting that newborn children suffer from a state of 'physiological' diabetes insipidus?

(A) The comparison would seem to apply in so far as the results of the present investigation suggest that the posterior pituitary-renal mechanism works imperfectly in the newborn child. Certain clinical evidence may be added to this. It is generally agreed that the function of the posterior pituitary antidiuretic hormone in the adult mammal is the conservation of body water especially under conditions of stress. There is evidence that it is difficult for some newborn infants, at least, to conserve body water. Infants aged 2–4 days are liable to a febrile disturbance (inanition or desiccation fever) which coincides with the period of the lowest fluid intake (Figs. 3, 5) [Faber, 1922; Kaufmann & Bickel, 1931]. This pyrexia has been shown to be accompanied by a diminution of the plasma water content [Bakwin, 1922]. Normal body temperature is quickly restored by an increase of fluid intake. The correlation between body hydration and temperature regulation is too well known to require comment [Herrington & Gagge, 1943], but further data on the water metabolism of newborn infants suffering from inanition fever are needed to make the supposition acceptable, that the rise in temperature is due, or partly due, to an inability to conserve water in the adult fashion, i.e. by the elaboration of a sufficiently concentrated urine. However, attention may be drawn to the similarity between the 'inanition' fever of the newborn child and the febrile response of cases of diabetes insipidus to water restriction [McGavack, Boyd & Gelvin, 1942].

(B) It should be pointed out that the comparison between the state of the water metabolism of the newborn infant and that obtaining in cases of experimental and clinical diabetes insipidus is not applicable in so far as the results of this investigation merely suggest that, compared with the normal adult, the renal tubules of the newborn infant are less sensitive to posterior pituitary extracts, whereas diabetes insipidus has been shown to be due to a deficiency of the posterior pituitary antidiuretic hormone. It appears quite possible that

in the newborn mammal such a hormonal deficiency coexists with the insensitivity of the renal tubules to the antidiuretic principle but experimental evidence on this point is lacking.

SUMMARY

1. Intramuscular doses of posterior pituitary extract which produced a pronounced inhibition of a water diuresis in adults had only a very slight and fleeting effect on the urinary concentration of newborn infants excreting a markedly hypotonic urine (Fig. 2). A low sensitivity of the renal tubules of the newborn children to the posterior pituitary antidiuretic hormone is thus indicated.

2. Freezing-point determinations of urine samples obtained from a series of infants aged 8 hr. to 6 days showed that these children excreted a comparatively concentrated urine during the first 2 days of extra-uterine life (Fig. 3). However, even these, most likely hypertonic, urines were still much below the average concentration of urines excreted by a group of normal adults (Fig. 4). Urinary concentrations were found to decrease rapidly during the 3rd day of life. Urine samples collected during the 4th, 5th and 6th days of life were, with few exceptions, markedly hypotonic.

3. The relation between the daily fluid intake and the concentration of urine samples obtained from the same group of newborn children (Fig. 5) suggests that the former may be an important determinant for the latter.

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THE DETERMINATION OF PLASMA VOLUME BY THE EVANS BLUE METHOD: THE ANALYSIS OF HAEMOLYSED PLASMA

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The determination of plasma volume by the Evans blue method requires the colorimetric analysis of plasma samples for the dye. In the method developed in this laboratory [Crooke & Morris, 1942], it was pointed out that haemolysis in the plasma samples interferes with the analysis, since haemoglobin is converted by the extraction process into a pigment (probably acid haematin) which has a significant light absorption in the same spectral region as Evans blue. The same objection applies to the butyl-alcohol extraction process of Harington, Pochin & Squire [1940], and also to the direct estimation of the dye in diluted plasma. In the latter case the interference is due to haemoglobin itself. By proper attention to the technique of blood sampling [Crooke & Morris, 1942], it is usually possible to reduce haemolysis to very low levels. However, in the investigation of certain pathological conditions, notably those involving circulatory collapse, withdrawal of blood samples can be very difficult, and in such cases the plasma may be considerably haemolysed. We have found that very slight haemolysis may cause an error of about 5% in plasma-volume determinations, and as the degree of haemolysis will vary from sample to sample, interpretation of dye elimination curves is difficult in such cases. Comparison with the direct method as described by Bonnycastle [1942] reveals that a similar error is present here also. The purpose of the present work was to develop a method in which this source of error could be eliminated. The most promising approach to the problem is the correction of the measured colour for the haem pigment present. The simultaneous colorimetric analysis of a solution for more than one light-absorbing component has been discussed by Vierordt [1873] and by others. Accurate analysis is only possible when the absorption maxima lie in different regions of the spectrum and it is usually necessary to correct for overlapping of the absorption curves. This is the case for the analysis of solutions containing Evans

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blue and haem pigment, and a brief account of the theoretical treatment is given here.

Let E_1^A, E_2^A be the extinctions at wave-lengths λ_1, λ_2 for concentration C^A of component A, and E_1^B, E_2^B be the corresponding extinctions at the same wave-lengths for concentration C^B of component B. Then if the Beer-Lambert law is valid for solutions of the two components

$$E_1^A = \alpha_1 C^A; \quad E_2^A = \alpha_2 C^A; \quad E_1^B = \beta_1 C^B; \quad E_2^B = \beta_2 C^B,$$

where α_1, α_2, β_1, β_2 are constants for a given depth of absorbing solution. If $E_1^{tot.}$ and $E_2^{tot.}$ are respectively the total absorptions at wave-lengths λ_1, λ_2, then,

$$E_1^{tot.} = E_1^A + E_1^B = \alpha_1 C^A + \beta_1 C^B, \tag{1}$$

$$E_2^{tot.} = E_2^A + E_2^B = \alpha_2 C^A + \beta_2 C^B. \tag{2}$$

By rearrangement of equations (1) and (2) it can be shown that

$$C^A = \frac{\beta_2 E_1^{tot.} - \beta_1 E_2^{tot.}}{\alpha_1 \beta_2 - \alpha_2 \beta_1}. \tag{3}$$

The denominator of this expression is a constant, and can be represented by K. Dividing equation (3) throughout by β_2 and rearranging, the following relation is obtained:

$$\frac{K}{\beta_2} C^A = E_1^{tot.} - \frac{\beta_1}{\beta_2} E_2^{tot.}. \tag{4}$$

In order to obtain C^A directly from this expression, it is necessary to evaluate α_1, α_2, β_1, β_2. If, however, the total extinctions at both wave-lengths are measured for a known concentration C^A of component A ($E_1^{tot.}$, $E_2^{tot.}$) and also for an unknown concentration C^A of the same component ($E_1^{tot.}$, $E_2^{tot.}$), the following relation can be derived from equation (4):

$$\frac{C^A}{C^A} = \frac{E_1^{tot.} - (\beta_1/\beta_2) E_2^{tot.}}{E_1^{tot.} - (\beta_1/\beta_2) E_2^{tot.}}. \tag{5}$$

In this case to determine the unknown concentration C^A, it is only necessary to evaluate the ratio β_1/β_2 for component B, a knowledge of the absolute values of α_1, α_2, β_1, β_2, and C^B being unnecessary.

The above discussion is, of course, only strictly valid for measurements made with monochromatic light, but provided the Beer-Lambert law relations hold for the concentration ranges in question, the conclusions can be applied to absorption measurements with light filters. The accuracy will be greatest when E_2^A/E_2^B is large or small compared with E_1^B/E_1^A, and this condition can be satisfied by the choice of suitable light filters. This has been done for solutions containing Evans blue and haem pigment. It has thus been possible to carry out satisfactory plasma-volume determinations with plasma samples which were considerably haemolysed. The method is obviously applicable to other dye methods of plasma-volume determination.

The determination of β_1/β_2 for haem pigment

An aqueous solution of haemoglobin was prepared by laking washed red cells in water and the haemoglobin concentration determined by the pyridine haemochromogen method of Rimington [1942]. Volumes of 0·1, 0·2, 0·3, 0·4, 0·5 ml. of this solution were added to 1·00 ml. portions of non-haemolysed plasma and the mixtures taken through the hydrochloric acid-alcoholic phosphotungstic acid procedure of Crooke & Morris [1942]. The extinctions of the solutions were measured in a single-cell photoelectric absorptiometer constructed in this laboratory. The measurements were made in 20 mm. cells
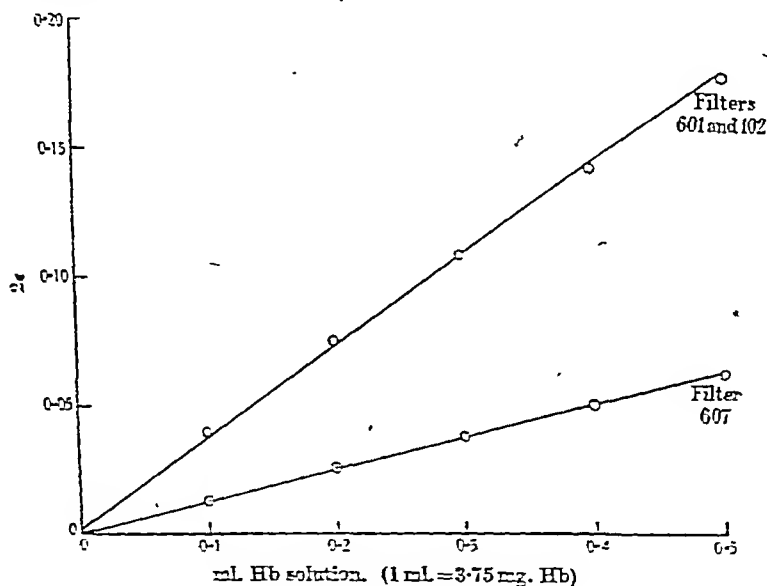

Fig. 1.

The determination of β_1/β_2 for control solutions

It was observed that the extinctions of control solutions measured against water in the two spectral regions showed a ratio close to the value of β_1/β_2 determined in the above experiment. Measurements were made for a series of five plasma samples all taken under conditions such as to minimize haemolysis. The results are given in Table 1, E_0 being the extinction in the orange and E_v the extinction in the violet.

TABLE 1

Sample	E_0	E_v	E_0/E_v
1	0·022	0·063	0·349
2	0·015	0·044	0·341
3	0·021	0·063	0·333
4	0·029	0·084	0·345
5	0·010	0·031	0·323
		Mean	0·338

The agreement of these values with β_1/β_2 as determined from Fig. 1 leads to two conclusions: first, the substance responsible for the control extinction obeys the Beer-Lambert law, and secondly, as the ratio E_0/E_v is so close to β_1/β_2, this substance is optically indistinguishable from haemoglobin. It is probable that the control extinction is actually due to very slight haemolysis, and that the plasma pigments do not pass into the alcoholic extract. In subsequent measurements water was used as the control and the extinctions calculated on the assumption that Evans blue and haem pigment were the only light absorbing substances present in the solution.

The estimation of Evans blue in the presence of varying amounts of haemoglobin

0·10 ml. amounts of a 0·14 % aqueous solution of Evans blue were added to 8·00 ml. samples of HCl-alcoholic phosphotungstic acid extracts of plasma containing varying amounts of haemoglobin. The extinctions of these mixtures were measured in the orange (E_0) and in the violet (E_v). The corrected extinctions ($E_{corr.}$) were calculated from the equation

$$E_{corr.} = E_0 - 0.343 E_v. \tag{6}$$

The values so obtained are given in Table 2.

TABLE 2

Exp.	Hb present (mg.)	E_0	E_v	$E_{corr.}$
1	0·37	0·304	0·117	0·164
2	1·12	0·321	0·157	0·167
3	1·49	0·335	0·204	0·165
4	1·87	0·345	0·227	0·167

The variation of the individual values of $E_{corr.}$ from the mean is within $\pm 1 \%$.

*The application of the correction to the determination
of plasma volumes*

The technique of injection of dye, withdrawal of blood samples and extraction of dye is identical with that originally described. A 1·00 ml. sample of undyed plasma, to which is added 0·100 ml. of a 1 : 50 dilution of the injected Evans blue solution, is taken through the extraction process, and is used as the standard. All solutions are measured in 20 mm. cells against a water control in both spectral regions. $E_{corr.}$ is calculated by equation (6) from the observed values of E_0 and E_r of each sample. Then if P is the plasma volume in ml., $E_{corr.}$ the corrected extinction of the solution measured, and $E_{corr.}^S$ the corrected extinction of the standard.

$$P = \frac{E_{corr.}^S \times 2500}{E_{corr.}}.$$

The derivation of the true plasma volume from the individual values has been discussed previously [Crooke & Morris, 1942].

SUMMARY

1. The error due to haemolysis of the analytical samples in plasma-volume determinations is discussed.

2. An optical method of correction for this error has been developed.

3. The practical application of this method to plasma-volume determinations is described.

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CHANGES OCCURRING IN PLASMA AND SERUM ON STORAGE AND THEIR PHYSIOLOGICAL EFFECTS

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Stored human blood, plasma and serum are now used extensively in the treatment of a wide variety of clinical conditions. Various workers—Lewisohn & Rosenthal [1933], Hughes, Mudd & Strecker [1938], Diggs & Keith [1939], Elliott, Macfarlane & Vaughan [1939], Girones [1939], Jorda [1939], Knott & Koerner [1939], Leedham-Green [1939], Lehmann [1939], McClure [1939], Patterson [1939], Aylward, Mainwaring & Wilkinson [1940], Black [1940], Brown & Mollison [1940], Brennan [1940], Brewer, Maizels, Oliver & Vaughan [1940], Clegg & Dible [1940], Edwards & Davie [1940], Ravitsch [1940], Scudder, Bishop & Drew [1940], Stewart [1940], and others—have reported on the use of whole blood, plasma and serum in human transfusion, the general consensus of opinion being that reactions to transfusion are infrequent, very slight, and, except in case of whole blood, do not increase with time of storage of the transfused material. Strumia, Wagner & Monaghan [1940 a, b] report favourable results with fresh and preserved plasma, but find that serum may cause severe reactions.

Landois [1875] suggested the transfusion of serum instead of whole blood. He injected a large number of heterologous sera into rabbits and dogs, and found that only those sera which agglutinated or haemolysed erythrocytes were toxic to recipients. Weiss [1896] described severe reactions on injection of serum from all species of animals into rabbits. Brodie [1900] found that cats were very susceptible to injections both of autogenous and heterogenous serum, and described a reaction to these injections which has since been known as the 'Brodie phenomenon'. All the sera investigated were toxic to cats, the doses required to produce a reaction varying from 0·1 to 10 c.c. Cats' blood withdrawn, defibrinated and reinjected within 2 min., he found to be toxic. Other animals, such as dogs, Brodie found to be almost insusceptible to serum. These results of Brodie's were in a large measure confirmed by the work of Ponder [1928]. Ponder found, however, that only 80% of the sera which he investigated were toxic to cats, and of these, only 50% showed a

high degree of toxicity. Ponder gives no information as to the ages of the various sera which he used. Doerr [1910] noted the similarity between anaphylactic shock and the toxic reactions to the injection of normal blood and serum. Zinsser [1911] described a toxic property of normal blood which was independent of its lytic and agglutinative properties. De Kruif [1917] and Novy & De Kruif [1917] found that fresh serum, injected as soon as possible after coagulation, was more toxic than old serum. They reported that plasma was non-toxic, but became toxic in the 'pre-clot' condition. The anaphylactoid shock produced by the injections was attributed to the production of an 'anaphylatoxin' in the animal's blood, in response to the injection of serum. Rous & Wilson [1918] evaluated the efficiency of the different fluids used as blood substitutes. They concluded that plasma was very good for this purpose, and that horse serum was effective in non-susceptible animals, e.g. dog and rabbit. Plasma and serum were more effective in restoring the blood pressure after haemorrhage than was Bayliss's gum-acacia solution. Bayliss [1920] investigated the reactions to transfusion, and suggested that the results of transfusion of incompatible blood were not due to haemolysis as such, but to a reaction to foreign serum protein, and that they were comparable to anaphylactic shock. Drinker & Brittingham [1919], reporting on the use of autogenous plasma and serum in transfusions, said that plasma which had been thoroughly freed from cellular elements by centrifugation or by filtration through porcelain was singularly non-toxic. In this respect, plasma differed markedly from serum. Levinson [1923] described an increased toxicity of plasma in pregnancy and in certain pathological conditions; this he attributed to an increase in the fibrinogen content of the plasma. Bond & Wright [1938] gave transfusions of lyophile serum to severely shocked dogs and found that this raised the blood pressure for several hours, but did not give recovery as the degree of shock was too great. Wright, Bond & Hughes [1938] used four times concentrated serum in doses of 4 c.c./kg. in order to reduce the cerebro-spinal fluid pressure in dogs. They found that the concentrated serum was effective for this purpose, and record no untoward results of the injections. However, Levinson, Neuwelt & Necheles [1940] reported severe reactions in 25% of dogs which they transfused with serum. Best & Solandt [1940], and Magladery, Solandt & Best [1940], found serum and plasma to be equally effective in restoring the blood volume of dogs shocked by histamine, trauma, trauma and haemorrhage, or haemorrhage alone. They took the precaution, however, of testing the sera to be used on the animals before shock was produced. Some samples of pooled dog serum did cause reactions. These were discarded and were not used for the attempted restoration of the shocked animals. Fine & Gendel [1940] gave dilute plasma (1–3 days old) to dogs with experimental intestinal obstruction. The survival time of these dogs was doubled when plasma was given in sufficient quantity

to compensate for the loss of fluid from the circulation. Buttle, Kekwick & Schweitzer [1940] found fresh plasma to be a good blood substitute in the treatment of acute haemorrhage in cats; no stored fluid plasma was used, but reconstituted plasma which had been dried by Dr Greaves proved fairly good. Plasma dried by Dr Davie killed the animal. When serum was used, severe reactions occurred in some cases. The authors noted the similarity between these reactions to serum and the Brodie phenomenon. They tried the effect of serum on dogs (which Brodie stated to be insusceptible) and noted a similar reaction in three cases. Reid & Bick [1942], investigating the pharmacological properties of serum, state that cats (but not dogs or rabbits) give severe reactions to the intravenous injection of homologous serum. They consider that the serum constituent responsible for this reaction is protein in nature, and is derived from the platelets.

We have not found any reference to work directed towards changes occurring in plasma or serum on storing, or the physiological effects of such storage. The work described below was undertaken when it was found that plasma or serum which had been stored 3–4 weeks in the blood bank was highly toxic to cats when injected intravenously in doses of from 0·5 to 2 c.c. whereas fresh human plasma or serum had no effect. Similarly, as reported below, fresh cat plasma is innocuous, whereas the same plasma becomes toxic on storage.

METHODS

Cats were anaesthetized with liquid 'Dial' usually injected intraperitoneally (0·6 c.c./kg.), but when intestinal plethysmography was attempted the anaesthetic was injected subcutaneously (0·7 c.c./kg.). Some cats were anaesthetized with chloralose or nembutal to rule out the effect of a particular anaesthetic; a few decerebrate preparations, decerebrated under ether anaesthesia 2–3 hr. prior to the experiment, were also used.

The trachea was cannulated and systemic arterial blood pressure was recorded from the carotid artery. In some cases pulmonary arterial pressure, and in some venous pressure (either from the jugular or splenic vein) was also recorded. Intrapleural pressures were recorded in some animals by means of a pleural cannula [Franklin & Gilding, 1932], and records were also obtained from intestinal and limb plethysmographs.

The human serum and plasma used for injection into the animals were samples of pooled plasma obtained through the agency of Dr W. H. P. Cant (Regional Transfusion Officer, Midland Region, No. 9) from the Army Blood Supply Depot, Bristol, the Ministry of Health Regional Transfusion Laboratory, Birmingham, the Ministry of Health Regional Transfusion Laboratory, Oxford, the Medical Research Council Blood Depot, Social Centre, Slough, and the Medical Research Council Serum Unit, Cambridge. Except where otherwise stated, the plasma or serum was of varying age, from 4 weeks

to 18 months. Doubtfully sterile or unduly cloudy samples were discarded. The cat plasma or serum was obtained and stored under sterile conditions. The serum or plasma was warmed before injection. The amount given was from 0·5 to 2 c.c. according to the size of the cat and the activity of the sample. Usually the injection was made into the internal saphenous vein in front of the ankle, to ensure thorough mixing of the plasma with the animal's blood, but some were made into the splenic vein or into the femoral artery.

RESULTS

The results of injection of active plasma or serum into the saphenous vein and into the femoral artery were similar, except that the reaction took slightly longer to come on when the injection was made by the arterial route.

Heart and blood pressure and peripheral circulation. Fig. 1 shows the effect of plasma on the heart and systemic blood pressure. Within 15 sec. the heart slows profoundly and. in a sensitive animal, may cease to beat altogether. The blood pressure falls almost to zero: cardiac massage and artificial respiration may be necessary in order to revive the animal. In some cases, after the cardiac inhibition has passed off, there are frequent dropped beats, indicating injury to the heart muscle. These findings agree with those of Brodie [1900] and Ponder [1928]. Records of pulmonary arterial pressure show that this rises as a result of plasma injection, the beginning of the rise being coincident with the beginning of the fall in systemic pressure (Fig. 2). Systemic venous pressure rises from about −4·5 to +4 cm. saline (Fig. 3). Portal venous pressure shows no significant change.

Limb volume increases after the injection. but the volume of the intestine decreases (Fig. 4). This diminution in volume depends on the mechanical emptying of the vessels as a consequence of the greatly increased peristalsis noted below.

Brodie described a vasodilatation in limbs and a vasoconstriction in the kidney as a result of serum injection.

Respiration. Respiration may cease altogether and artificial respiration may be necessary in order to revive the animal, or there may be spontaneous recovery. In the latter case. periods of apnoea are interposed between two or three gasps, and eventually normal respiration is resumed (see Fig. 5). Records of intrapleural pressure show a rise in the base line of the water manometer, indicating a decrease in the negative pressure in the thorax. This decrease in negative pressure is probably the result of an accumulation of blood in the thorax. Fig. 5, which is typical of all the intrapleural pressure records which were made. shows that for each inspiration after plasma or serum injection a greater negative pressure is required. This increase in negative pressure is doubtless the result of bronchial constriction, since the injection of adrenaline brings about an immediate reduction in the negative
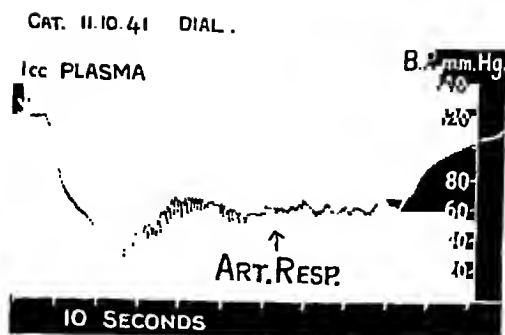
Fig. 1. Effect of 1 c.c. stored plasma on heart rate and arterial pressure.
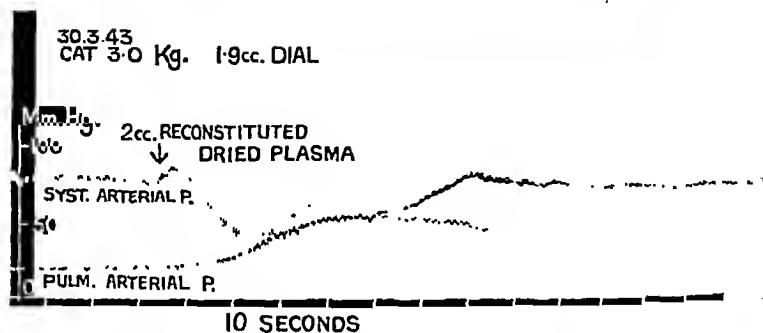

Fig. 2. Effect of 2 c.c. reconstituted dried plasma on heart, systemic and pulmonary arterial pressures. Normal respiration.
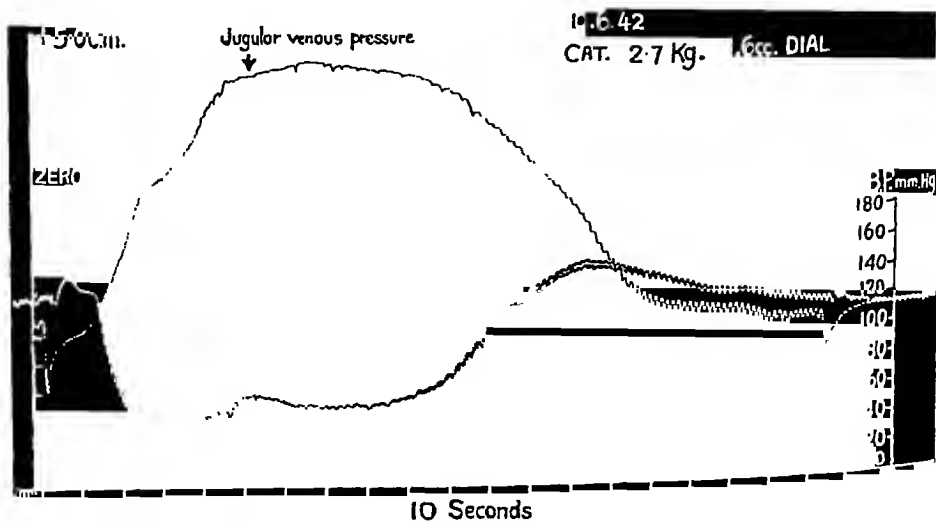

Fig. 3. Effect of 2 c.c. stored plasma on jugular venous pressure and systemic arterial pressure.

pressure required for inspiration. The effect of the adrenaline, however, soon wears off. The variations in intrapleural pressures do not return to normal

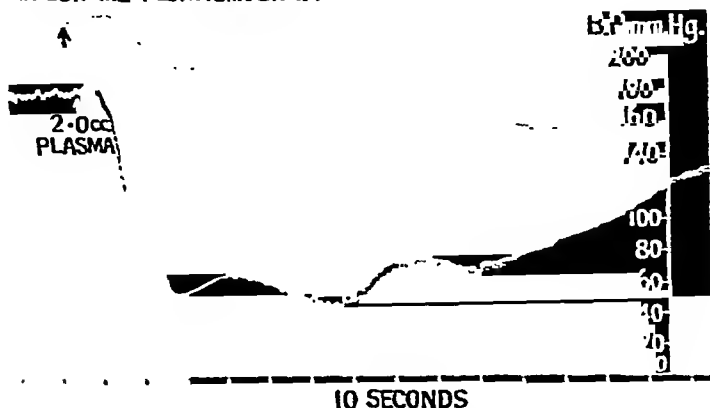**22 5 42 . CAT 2·0 Kg. 1·2cc DIAL**

INTESTINAL PLETHYSMOGRAPH

Fig. 4. Effect of 2 c.c. stored plasma on intestinal volume and systemic arterial pressure.
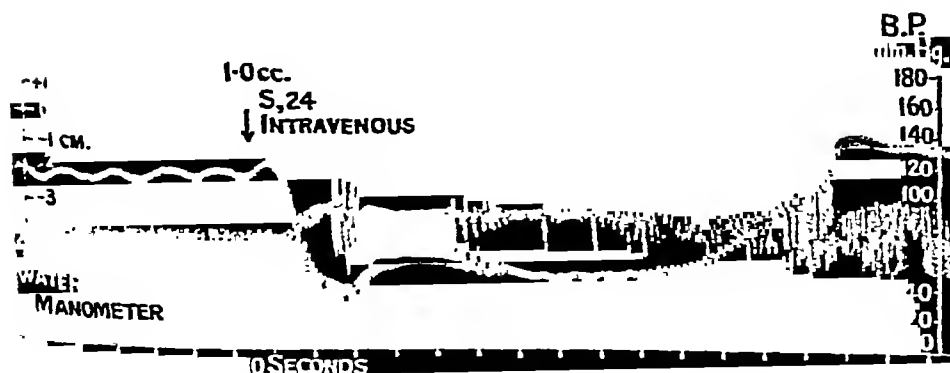
Fig. 5. Effect of 1 c.c. of stored serum on intrapleural and systemic arterial pressures. The oscillation of manometer during apnoeic periods is due to transmitted heart pulsations.

until a period of 10–20 min. has elapsed. In some cases rapid shallow respirations precede the complete cessation of respiration.

Alimentary canal. The intestines, examined either through an abdominal wound, or in the intestinal plethysmograph, show greatly increased peristalsis.

Animals with intact abdomens produce noticeable borborygmi. Retching may occur, and if the animal has been fed, vomiting. In decerebrate animals defaecation occurs.

Bladder. Micturition occurs if urine is present.

Eyes. Within 10–15 sec. of the injection, the nictitating membrane retracts, the palpebral fissure widens and the pupils dilate slightly. The dilatation is immediately followed by an intense pupillary constriction, the pupil being reduced to a mere line. This condition lasts for from 2 to 5 min., when the nictitating membrane, palpebral fissure and pupil return to the pre-injection condition. Usually these signs are accompanied by the secretion of tears. In some animals the constriction of the pupil is immediate and is not preceded by dilatation.

Saliva. Obvious salivation has never been observed.

Sweating. There were no signs of sweat on the animals' pads.

Movements. In anaesthetized animals there was marked opisthotonos, flattening of the pinnae and stretching movements of the limbs. In decerebrate animals, in addition to opisthotonos, the limbs performed co-ordinated rapid running movements for a period of about 2 min. after the injection.

Autopsy. The most striking pathological changes were seen in the lungs, where there were petechial haemorrhages and occasional areas of congestion. Portions of the lung were sectioned, and showed in some parts extravasation of blood into the connective tissue, and, in isolated patches, rupture of the alveolar walls with haemorrhage into the alveoli. The pulmonary vessels, particularly arterioles and capillaries throughout the lungs, showed an abnormally large number of leucocytes of the polymorphonuclear type. That this finding was the result of injecting stored plasma or serum was proved by removing a lobe of lung, closing up the thorax by suture before giving plasma, and then removing another lobe at autopsy after injecting plasma. Examination of subsequent wax sections of these two lobes showed a normal appearance in the lobe removed before plasma injection, while the section of lung after injection showed the usual picture seen in Pl. 1, fig. 6. In experiments in which the animals were left up to 7 hr. before being killed, the lungs still contained abnormal numbers of polymorphonuclear cells inside vessels.

The suprarenals showed little macroscopic damage, but occasionally there were patches of haemorrhage in the cortex. Wax sections showed numerous polymorphonuclear cells in the small vessels, more particularly in the cortex. This effect was controlled by removing a suprarenal before injecting the plasma. Here, as in the lung, the accumulation of polymorphonuclear cells was shown to be the result of the plasma injection (see Pl. 2, figs. 7 *a*, *b*). The liver appeared normal but here again an increased number of leucocytes was seen in section. The accumulation of white cells was, however, not so marked as in the lung and suprarenal.
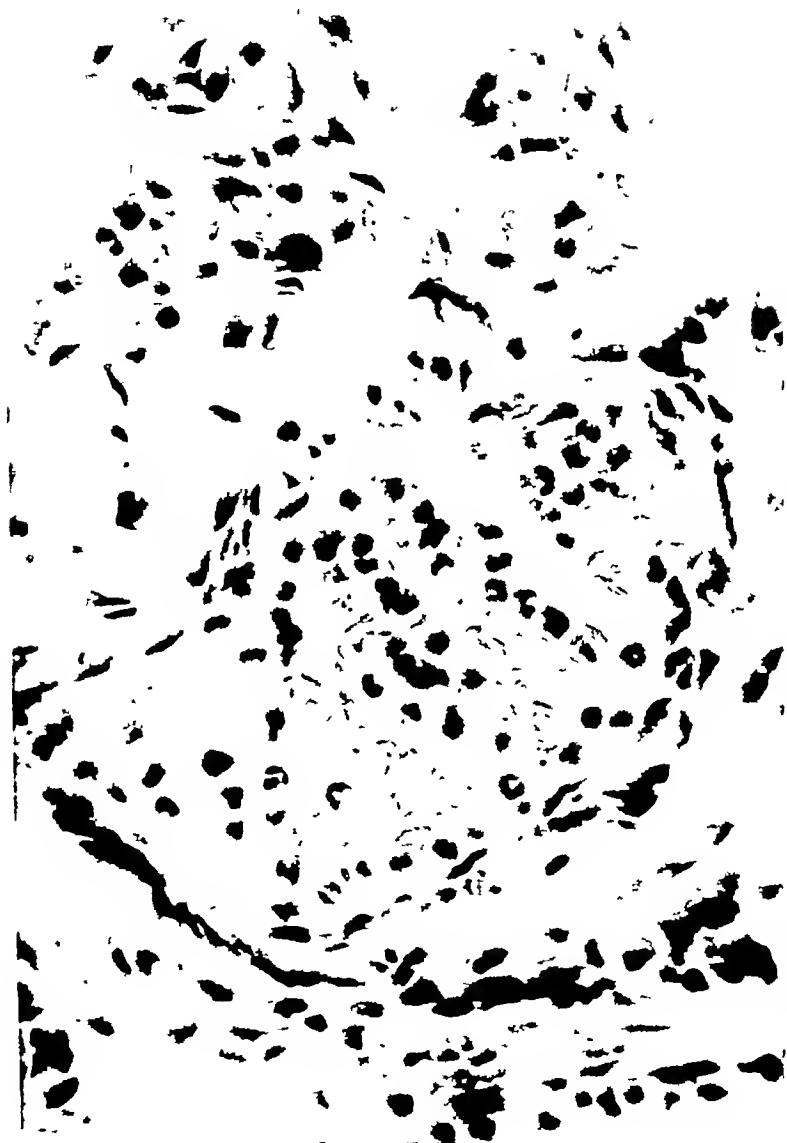
Fig. 6. Lung removed at autopsy after plasma, showing numerous polymorphonuclear leucocytes in lumen of arteriole.
Wax section H. and E. ×520.

PLATE 2   THE JOURNAL OF PHYSIOLOGY, VOL. 102, No. 4

Fig. 7b. Wax section H. and E. of suprarenal removed at autopsy after plasma, showing capillary packed with polymorphonuclear leucocytes. × 1000.
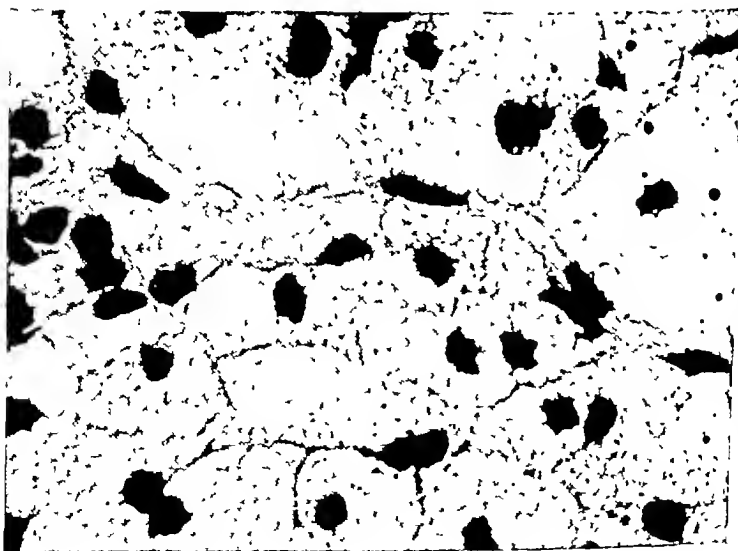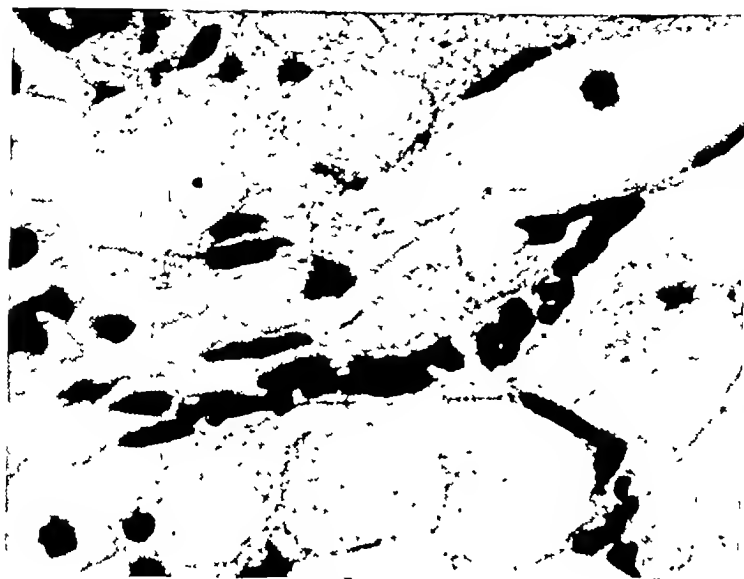

Fig. 7a. Wax section H. and E. of suprarenal removed before injection of plasma, showing capillary with many red blood cells but no leucocytes. × 1000.

Other tissues, such as spleen, kidney, intestine, skeletal muscle and diaphragm, showed no macroscopic or microscopic abnormalities.

Blood changes

The above histological findings raised the question of the leucocyte content of the blood before and after plasma injection. For this investigation a bleeding cannula was inserted into a femoral artery, and blood was taken before and after giving plasma. In order to correlate these findings with the other actions of plasma, samples of blood were usually taken between 30 and 40 sec. after giving the injection. Examination of the blood revealed a pronounced leucopenia, mainly, though not entirely, due to a diminution of polymorphonuclears. A protocol, which is typical of all experiments whether human or cat serum or plasma was injected, is as follows:

22 June 1942. Experiment 54. Cat 3·3 kg. 1·7 c.c. Dial intraperitoneally

	Before injection		After injecting 2 c.c. cat serum (collected 13 May 1942)	
Total white cells ...		6000		3100
Polymorphs	50%	3000	17%	527
Lymphocytes	37%	2220	66%	2046
Eosinophils	12%	720	13%	403
Basophils	1%	60	3%	93
Monocytes	—	—	1%	31
Haemoglobin	67%	—	62%	—
Haematocrit	37·6% (cells)	—	35%	—

After the initial leucopenia, the white cell count rose, and after 5–6 hr. was three or four times as high as the pre-injection level, the leucocytosis being mainly due to youthful polymorphonuclear cells; a number of nucleated red blood cells were seen, but no counts of these were made. The white cell count has not been followed for more than 7 hr. after plasma injection.

The injection of fresh human plasma had no action on leucocytes; the white blood cell count before injection was 8150 and after 7900, and the polymorphs 42 and 44% respectively.

The above experimental findings of the effect of stored plasma in the cat suggest: (a) stimulation of the parasympathetic nervous system either peripherally, centrally or reflexly; (b) stimulation of the central nervous system; or (c) a generalized chemical or physical action on the systems of the body. The following experiments were designed therefore to localize the site or sites of action of stored plasma.

Effect of atropine. Injection of plasma into the atropinized animal (atropinization tested with 5–10 μg. of acetylcholine) produced a fall in blood pressure, but no cardiac inhibition. The fall was not so marked as in the normal animal, and recovery was more rapid (Fig. 8). The effect on respiratory rate was similar to that in the non-atropinized animal, but there was no bronchial constriction, and the rise in the general baseline of the intrapleural pressure did not occur (Fig. 8). This suggests that the rise in intrapleural pressure

referred to earlier is the result of cardiac inhibition, which brings about an accumulation of blood in the thorax. The limb volume increased, and the intestinal volume—which in the non-atropinized animal diminished markedly —also increased slightly. This finding suggests that plasma may have a direct dilator action on blood vessels. After atropinization, plasma had no action on the intestinal muscle; no peristalsis was observed, neither defaecation nor vomiting, and there was no micturition. The pupils constricted slightly as a result of plasma, although acetylcholine had no effect on them. This constriction may possibly have been due to a central inhibition of sympathetic
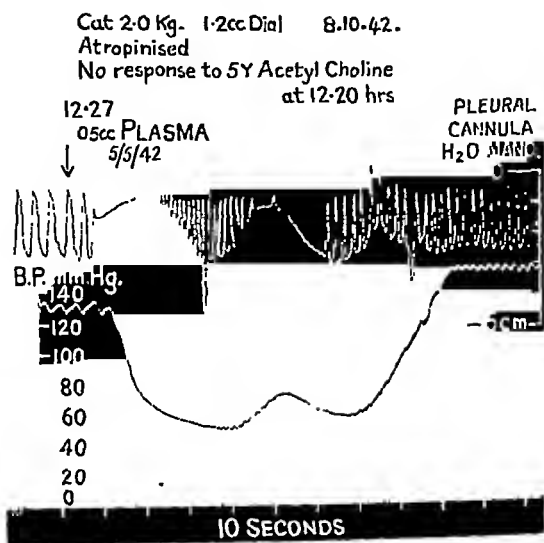

Fig. 8. Effect of 0·5 c.c. stored human plasma on heart rate, blood pressure and intrapleural pressure in the atropinized animal.

tonus. Opisthotonos and stretching of the limbs was diminished by atropine. The changes in the white blood cells were not influenced by atropinization of the animal.

Effect of section of the vagus nerves. Section of the vagus nerves in the neck abolished the cardiac inhibition, but there was still a fall in blood pressure, although this was less than when the vagi were intact. Fig. 9 shows the effect of cutting the vagi during the reaction. As far as respiration was concerned, section of the vagi abolished the changes in rate produced by plasma, but bronchial constriction was even more marked. Fig. 10 shows this effect, and shows also that there is no rise in the base line of the intrapleural pressure such as occurs with intact vagi. It also shows the effect of adrenaline on the bronchial constriction. Plethysmographs record an increase in limb and intestinal volume. The intestines no longer showed increased peristalsis. There

was no defaecation, vomiting or micturition and no opisthotonos or limb movements.
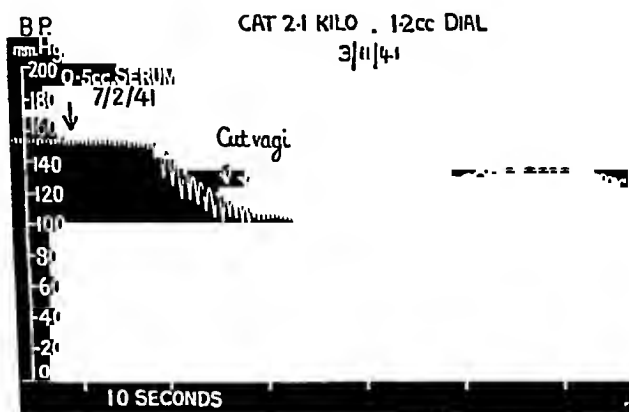

Fig. 9. Effect of vagal section on heart rate and blood pressure during response to injection of 0·5 c.c. of stored human serum.
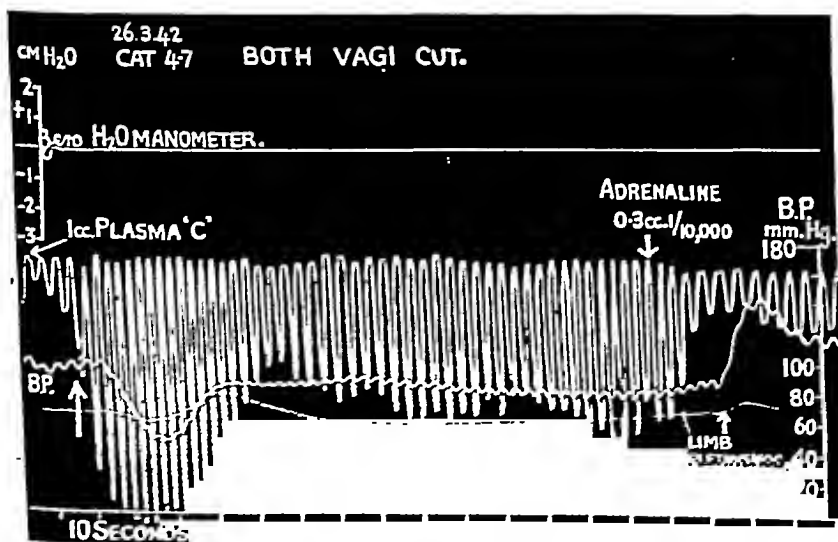

Fig. 10. Effect of 1 c.c. of stored human plasma on intrapleura pressure, heart rate, blood pressure and limb volume in spinalised cat, and injection of adrenaline, 0·3 c.c. of 1/10,000 during the response.

Bain, Irving & McSwiney [1935] and by Irving, McSwiney & Suffolk [1937] as a result of stimulation of vagal and sympathetic nerves from the abdominal viscera.

Brodie [1900] showed that the site of action of serum was on afferent vagal endings in the lungs, and that the response to its injection was a reflex phenomenon. This he attempted to prove by cutting the vagal fibres running to the lungs, but, as he points out, the operative disturbance and subsequent necessary artificial respiration interfered considerably with the reaction. Brodie states, in fact, that the reaction cannot be obtained successfully from cats under artificial respiration, and that it is necessary to switch off the pump in order to observe it; we have not found this, probably on account of the different method used for ventilating the animal. Cats under artificial respiration show the vascular and cardiac reactions characteristic of plasma if the nervous system is in reasonable condition. However, as the phenomenon is largely a reflex one, cats which have suffered severe anoxia of the central nervous system as a result of respiratory failure will give very unreliable results.

Owing to the difficulties involved in section of the pulmonary vagus, we attempted to show whether the phenomenon was reflex or central in origin by means of cross-circulation experiments. The arterial connexion between carotid arteries presents little technical difficulty. The external jugular veins, however, constrict to such an extent that it is difficult to insert a cannula big enough to ensure an adequate venous drainage. This difficulty, however, can be overcome in part by exposing two areas on each external jugular vein, about 2 cm. apart, by snipping off the skin over the vein with scissors and leaving the exposed connective tissue and vein unprotected. The necessary ligatures are inserted deeply under the vein (which should not be dissected) by curved surgical needles, and left *in situ* until wanted. The exposed surfaces dry somewhat and, when the time comes to cannulate, the vessel does not contract to anything like the extent which it does without this precaution.

Two animals of approximately the same size were chosen, and both carotid arteries and external jugulars of each prepared for cannulation; the internal jugulars were tied. A strong cord was put in position around the vertebral column, at the level of the first and second cervical vertebrae, to occlude the vertebral arteries [Sherrington, 1919]. Then 17 mg. of heparin was injected intravenously into each animal. The animals were placed side by side and the neighbouring jugular veins cannulated proximally and distally and joined with rubber tubing, so that blood from each animal's head flowed into the other's heart. Next both carotids were joined in a similar manner. Then the outside jugular veins were similarly treated, and finally the vertebral arteries were tied off.

The blood pressure was recorded from the femoral artery in each animal. Fig. 11 shows the effect of an injection of 1 c.c. of stored human serum into an internal saphenous vein in cat A. It will be seen that the cardiac effects and fall of blood pressure occurred in the animal receiving the injection, in spite of the fact that its head was receiving blood from cat B's systemic circulation.

The efficiency of the cross circulation was tested by injecting 3 c.c./kg. of a 4 % solution of bromphenol blue intravenously into cat A. Rous & Gilding [1929] demonstrated that this quantity of dye stains the mouth in less than

a minute, and in 3 min. a maximal staining of the whole animal was reported. In the present experiments within 30 sec. of the dye injection, it appeared in the mucous membranes of the mouth of cat B, and within 3 min. the mucous membranes of nose, mouth and eyes were heavily stained, whereas the injected cat's own head had the merest tinge of the dye after 3 min. This experiment therefore confirms Brodie's opinion that the phenomenon is reflexly produced. However, it is seen from the results of cervical vagal section described above that the vagus cannot be the only sensory nerve involved in the reflex.
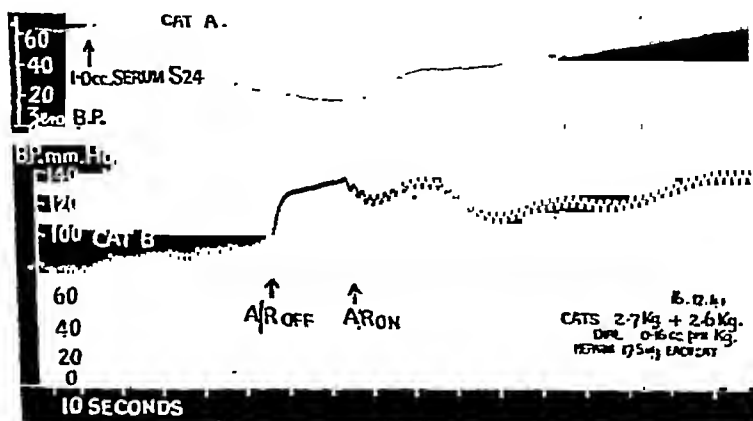

Fig. 11. Crossed circulation of animals' heads. Injection of 1 c.c. of stored human serum into saphenous vein of cat A. (Cat A was breathing normally; cat B was artificially ventilated, and during the crucial experiment the pump ceased working in between the two arrows.) Frequency of heart beat of cat A fell from 40 to 19/10 sec.

Attempts were made to determine the precise location of the vagal nerve endings in the lung concerned in the reflex. Brodie & Russell [1900] showed that inhalation of chlorine, bromine or chloroform vapour, in the dog or cat, could initiate a vagal reflex comparably with the injection of serum. A fine cloud of plasma was sprayed over the tracheal cannula of a cat by means of a 'Mistol' paint sprayer. This had no effect of any kind on the animal, so that the nerve endings in the alveoli of the lung cannot be chemoreceptors sensitive to inhaled plasma. McLaughlin [1933] described an unusually rich distribution of vagal nerve endings in the cat's visceral pleura. It was considered possible that these numerous endings were responsible for the cat's peculiar sensitivity to injections of stored plasma and serum, but the intrapleural injection of stored plasma was without effect.

Leucocytes. The presence of pathological changes in the lung, and the great increase in the number of polymorphonuclear leucocytes in the lung vessels suggested that the clumping of leucocytes might produce minute pulmonary emboli, and thus reflexly cause the cardiac and respiratory changes. Reflex

slowing of the heart, and respiratory effects similar to those produced by plasma injection, have been described by Dunn [1920] and by Binger & Moore [1927], Binger, Boyd & Moore [1927] and Binger, Brow & Branch [1924–5] as a result of multiple minute pulmonary emboli. The emboli were produced in these cases by the injection of starch grains or various plant seeds into the pulmonary circulation.

It was at first thought that plasma influenced the leucocytes in such a way as to make them adhere severally to the walls of capillaries. If, however, the injection was made into the femoral artery, the only difference from intra-venous injection noted was a delay in the onset of the symptoms. In order to test the embolism theory still further, recourse was made to the injection of dialysed Indian ink (Higginson's American Drawing Ink, non-waterproof). Rous & Gilding [1929] showed that Indian ink agglutinates *in vitro* and *in vivo* when mixed with plasma. Three c.c./kg. of this ink was injected intravenously and produced no response. Finally, when plasma was injected into the splenic vein, it had no effect on the heart, blood pressure, respiration, etc. but still produced a leucopenia. The missing leucocytes were found when sections of the lungs were examined. This last experiment shows that the sticking of leucocytes within the lung is not responsible for the reflex response to plasma injections; further, that the liver can protect the animal against the toxic substance in stored plasma.

Brodie [1900], quoted by Ponder [1928], demonstrated that one injection of plasma desensitizes the animal for a time (about 40 min.). We have found that this desensitization disappears after an interval of 30–35 min. An in-jection thereafter will produce all the phenomena described above except the leucopenia, which, however, still persists from the previous injection. The leucocyte count does not return to the pre-injection level until an interval of about 2 hr. has elapsed. Leucocyte counts made 35 min. after plasma are frequently lower than those determined immediately after the injection. This is further proof that the leucopenia is not the cause of the other effects.

Experiments in cross-precipitation with stored human plasma and cats' plasma showed that no precipitins exist in either blood for the proteins of the other. Also, even had precipitins been present in the cat/human plasma, this would not have explained the activity of the stored cat plasma. Cats' red cells were not agglutinated by stored human plasma.

In the above experiments the plasma or serum was injected rapidly into the vein within 2–3 sec. It was decided therefore to try the effect of injections of plasma at a rate more comparable with that of human transfusions. When plasma is run into a vein at a rate of 0·6 c.c./kg./min. for 20 min., the effect on the various systems is much less than that described above, but still there is marked fall of blood pressure and cardiac inhibition at the beginning of the transfusion. The blood pressure rises gradually, but is usually lower at the

prevailing at the Queen Elizabeth Hospital, citrate being used as the anti-coagulant. Half of the blood was put into the blood bank at the hospital. The other half was centrifuged aseptically at 3000 r.p.m. for 20 min. to remove the corpuscles. A sample of the plasma was then injected into a cat
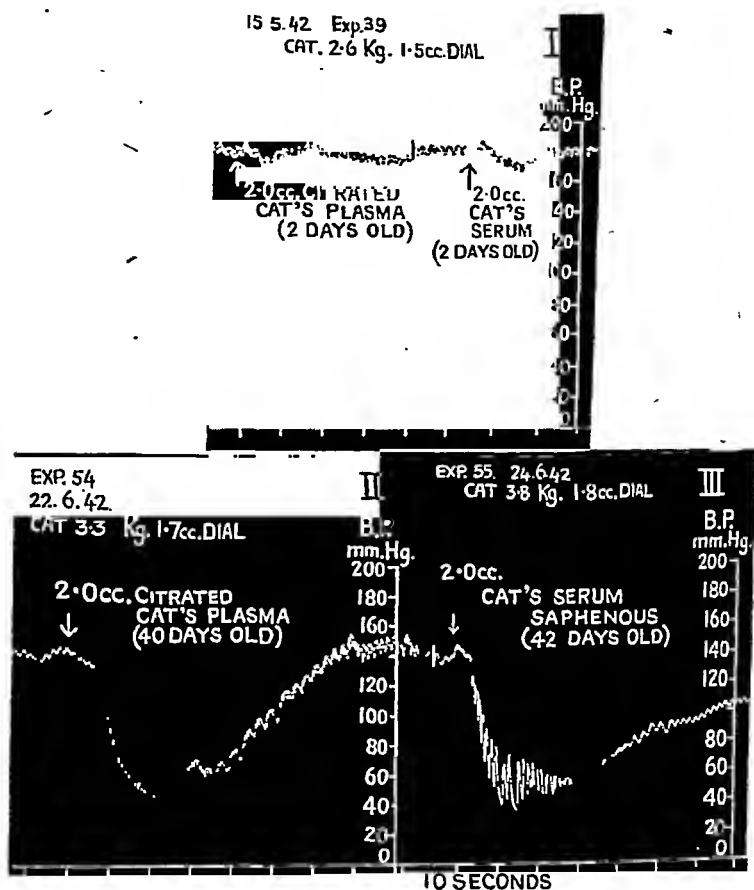
Fig. 13. Development of toxicity in citrated cats' plasma or serum. I. Effect of 2 c.c. cat serum and plasma, 2 days old. II. Effect of 2 c.c. cat plasma, 40 days old. III. Effect of 2 c.c. cat serum, 42 days old.

within 45 min. of bleeding. Injections of 0·5, 2 and 10 c.c. of this citrated plasma were completely inactive. The remaining plasma was then decanted aseptically into sterile vessels and stored with the whole blood at the blood bank at the hospital. This plasma and whole blood was thus exposed to the same conditions as that which has been obtained from time to time from stocks at the blood bank. No toxicity had developed in 7 days, a slight toxicity in 14 days, and maximum toxicity in 24 days (see Fig. 12). The

citrated whole blood behaved in the same way as the separated plasma; this differs markedly from the findings with heparinized plasma reported below.

To rule out the effect of the citrate used as an anticoagulant, some blood collected from the same donor at the same time as the above was made incoagulable with sterile heparin (4 mg. in 4 c.c. of saline per 100 c.c. of blood). The subsequent treatment was identical with that for blood collected into citrate.

It will be seen from Fig. 12 (III) that the separated heparin plasma became very toxic within 7 days. We can offer no explanation of this unexpected result. No toxicity developed in the heparinized whole blood until 3 weeks had elapsed, when signs of coagulation appeared.

Subsequently, samples of pooled plasma of different ages (from 7 to 28 days old) were supplied by Dr W. H. P. Cant (Regional Blood Transfusion Officer). The pooled plasma, like that from a single donor, is non-toxic until it is about 21–23 days old.

Stored cats' plasma behaves in a similar way to human plasma, except that it becomes toxic rather more quickly, full toxicity being developed in 2–3 weeks (see Fig. 13).

As stored human serum had been found to be toxic to cats, it was at first thought that the toxicity which develops in stored plasma might in some way be connected with the clotting mechanism, as the heparinized whole blood became toxic at the same time as clotting occurred.

Serum was prepared by recalcification of citrated whole blood and plasma less than 14 days old. This serum was found to be entirely without effect on the animal. Both human and cats' bloods behaved similarly in this respect. Cat's serum, obtained from clotted fresh blood, or by centrifugation of defibrinated blood, develops toxicity at the same time as plasma obtained from the same blood, i.e. within 2–3 weeks.

It is proved therefore that fresh plasma or serum, either human or cat's, is non-toxic to the cat. When the same preparations are tested over a period of one month, a toxicity is found to develop.

Investigation of toxic constituent

Investigations were made with the object of discovering the nature of the toxic constituent of stored plasma and serum.

The test for activity of preparations was the production of a typical response (described above) on their injection into the saphenous vein of the cat. The doses for injection were calculated in terms of the volume of the original plasma from which a given fraction had been prepared.

Histamine extracts. The stored plasma was tested for the presence of histamine by means of extracts made by Barsoum & Gaddum's method [1935].

Fractionation by ammonium sulphate. The plasma proteins were fractionated by the addition of saturated solutions of ammonium sulphate. The ammonium sulphate solution was added to the plasma in the calculated volumes required to produce various percentage saturations; for

full saturation, solid ammonium sulphate was added to the plasma until undissolved crystals remained at the bottom of the vessel.

At each stage of saturation the precipitate was filtered off, sucked as dry as possible on a Buchner funnel, dissolved in water, and dialysed to remove the remaining ammonium sulphate.

Fractionation by lead acetate. Plasma or serum was brought to pH 5·0 by the addition of dilute acetic acid, the pH being estimated by means of a glass electrode and the Cambridge pH meter. Lead acetate in 10% solution was then added in the proportion of 10/100 c.c. of plasma or serum. The pH was then raised by stages, by the addition of NaOH to 6·0, 6·5, 7·0 and so on to pH 9·0, by which time all the protein had been precipitated. At each stage the precipitate was centrifuged off and taken up in Sorensen's phosphate buffer, pH 8·0, the precipitate from 100 c.c. of plasma being taken up in 20 c.c. of buffer. The lead was precipitated as lead phosphate and the protein went into solution. The precipitated lead was removed by centrifugation, and a suitable dose of the protein solution was injected into an animal in order to test its activity. The phosphate buffer itself had previously been found to be non-toxic to the animal.

The lead fractionation was used both on the original plasma and serum and on protein solutions prepared by fractionation with ammonium sulphate.

Fractionation by metaphosphoric acid. In order further to fractionate the proteins of the plasma, or possibly to elute some adsorbed non-protein substance [Rimington, 1941], solutions of $3N$ metaphosphoric acid were used. The metaphosphoric acid was added to bring down the pH of the solution in stages to pH 2·0 by which time all the protein in the solution had been precipitated. At each stage the precipitated protein was centrifuged off and taken up in saline, to which a few drops of NaOH had been added to neutralize the acid. The solutions of protein so produced were tested for activity in the usual way.

RESULTS

The extracts of plasma made by Barsoum & Gaddum's method caused only a slight and transient fall of blood pressure in the cat. Further proof that the reaction is not due to the presence of histamine in the plasma, is given by the fact that an injection of histamine gives no protection against an injection of plasma made immediately afterwards. If, however, two injections of equal doses of histamine are made successively into a cat, the second injection produces a much reduced response.

When serum was dialysed overnight against saline, the dialysed serum was active, but the dialysate completely inactive (Fig. 14). Ultrafiltration of stored plasma produced an active ultraconcentrate and an ultrafiltrate which had only a slight depressor activity. Thus it seemed that the toxic substance must be a large molecule or must be adsorbed on to a large molecule.

Plasma was dialysed against a continuous flow of Birmingham tap water (which is almost as pure as distilled water). The precipitated euglobulin was filtered off, taken up in saline, and a portion injected; it proved completely inactive. The dialysed plasma was taken to pH 5·3 when a further precipitate of globulin occurred. This, too, proved completely inactive when dissolved in saline and injected. The whole of the activity was still present in the proteins remaining in solution.

The precipitate produced by half-saturating stored plasma with ammonium sulphate was filtered off, taken up in distilled water and dialysed; it was inactive. However, when the filtrate from half-saturation was dialysed and

injected, it was found to have an activity equal to that of the original plasma, when injected in a comparable dose. Attempts were made to fractionate the albumin further by means of ammonium sulphate. It was found possible to bring down all the active substance between 66 and 75% saturation with the salt. The active fraction was further purified by precipitation with lead acetate; the activity went with the proteins precipitating between pH 6·5 and 7·0. The protein content of this final active fraction estimated by Kjeldahl's method was found to represent only 8% of the original serum proteins.
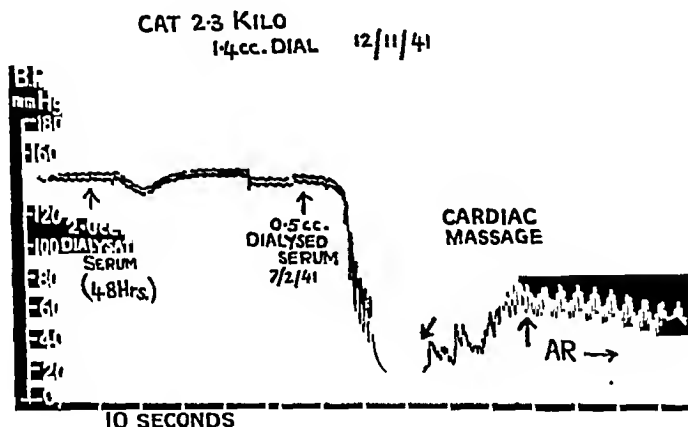
Fig. 14. Activity of dialysed serum and serum dialysate.

The active protein fraction prepared by the lead treatment could not be further purified by treatment with metaphosphoric acid. The active fraction came down between pH 3·6 and 2·6. By treatment of the original plasma with phosphoric acid, an active fraction could be separated over the same pH range.

It seems therefore that the substance responsible for the toxic action of stored plasma and serum is a protein of the albumin class. This protein is precipitated between 66 and 75% saturation with ammonium sulphate; between pH 6·0 and 7·0 by the lead acetate treatment; and between pH 3·6 and 2·6 by treatment with metaphosphoric acid. The active protein represents not more than 8% of the total protein of the original serum or plasma. This protein gives a strong Millon's reaction and a feeble glyoxylic reaction.

Experiments on other animals

Cats proved to be sensitive animals for estimating and analysing the changes that occur when plasma or serum is stored, but it was of interest to see if any of these reactions could be obtained with other animals. For this purpose,

a few experiments were done on dogs, rabbits and human beings with the following results.

Dogs. Doses comparable to those in the cat had little effect, but if 20–50 c.c. of stored plasma was injected intravenously during the course of 1–2 min.
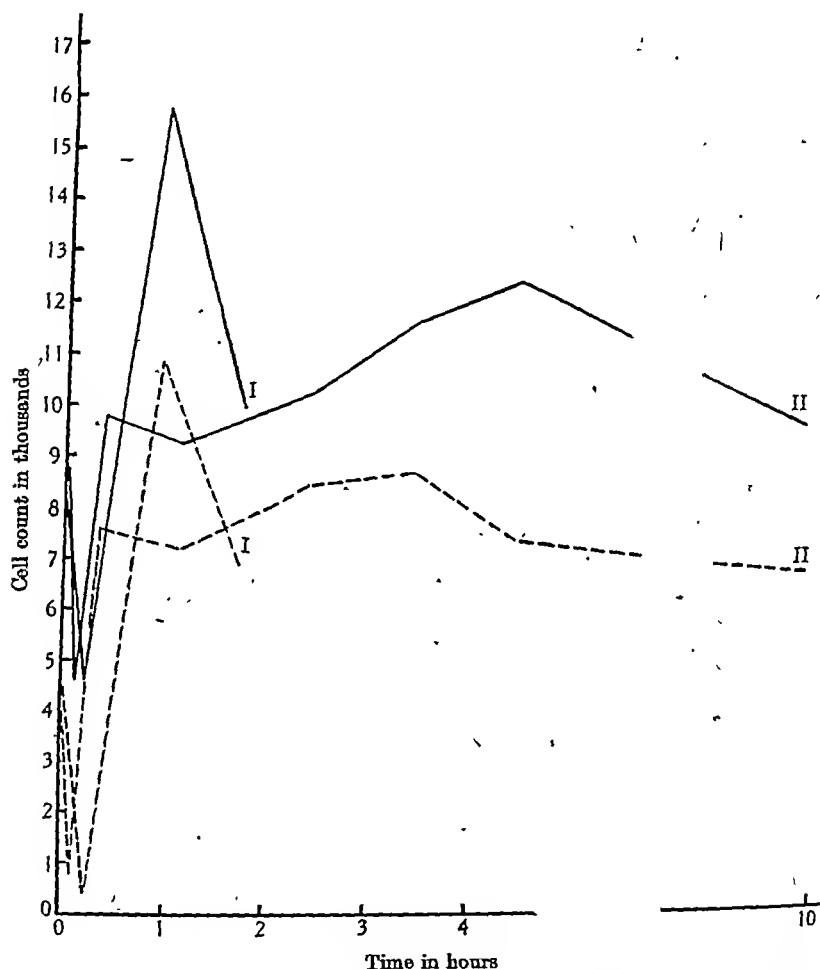
Fig. 15. Continuous lines—total leucocyte count. Dotted lines—polymorphonuclear count. I. Subject—R.A.M.C. II. Subject—H.P.G.

into dogs of 7–15 kg., the blood pressure fell to about 50–70 mm. Hg, returning to normal in 2–3 min. The heart rate was unaffected, but occasional dropped beats occurred. Respiration may cease altogether, or become rapid and shallow for several minutes. There was no micturition, defaecation, opisthotonos or pupillary reaction. There was a slight leucopenia followed by leucocytosis. At autopsy, small congested areas were to be seen

and microscopically the arterioles and capillaries were crowded with polymorphonuclear cells.

Rabbits. Intravenous injections of 2 c.c. stored human plasma had no demonstrable action, except for a momentary slight leucopenia, followed by a pronounced leucocytosis, the cell count returning to pre-injection level in the course of 3–4 hr. The intravenous injection of 20 c.c. of stored plasma in the anaesthetized rabbit had no immediate effect on heart rate or blood pressure; respiration became rapid and shallow; there was no micturition, defaecation, opisthotonos or pupillary reaction. Death occurred after 80 min. The white blood cell count fell from 6750 to 1650 and polymorphs from 4050 down to 82.

Man. Stored plasma known to be toxic to cats was injected into two subjects as follows:

R.A.M.C. (72 *kg.*). 60 c.c. injected into antecubital vein during the course of 20 sec. No effects on respiration or heart rate were observed. The subject said he had 'a muzzy sensation like early anaesthesia, which soon passed off'. He had intense headache next day. The effects on white blood cells are shown in Fig. 15.

H.P.G. (62 *kg.*). 180 c.c. injected into saphenous vein during 2 min. had no effect on the heart rate recorded from the opposite leg, but a few dropped beats occurred; respiration recorded by stethograph showed no effect. During the injection, the subject felt and looked flushed, his lips and tongue felt numb and swollen and his throat dry for 2–3 min. He felt no nausea, but had headache for 24 hr. and general malaise and loss of appetite for 48 hr. The effects on white blood cells are shown in Fig. 15.

DISCUSSION

The above experiments show clearly that a change occurs in plasma or serum when it is stored for upwards of 3 weeks. The nature of this change whether physical or chemical is obscure, but that it is associated with the development of a substance extremely toxic to the cat is proved. The substance appears to be a protein belonging to the albumin class, or else is firmly adsorbed on to such a protein. Brodie [1900] obtained a similar reaction with serum, and stated that this active substance was only produced when blood clots, and also that the interaction of blood corpuscles was a necessary condition for its formation. Neither of these views is tenable in the light of our experiments. The stored plasma and serum used here have been subjected to filtration by Seitz filters which remove all cells and platelets, and yet plasma, without clotting, has been shown to become toxic on storage. Similarly, fresh citrate plasma does not become toxic when clotted by the addition of calcium, a fact which puzzled Brodie. Reid & Bick [1942] suggest that the toxic substance in serum is a protein which they state arises from platelets. This is unlikely

for the following reasons: (1) Plasma which has been passed through a Seitz bacterial filter, and therefore contains no platelets, develops toxicity on storage. There is no difference between the time of development in filtered and unfiltered plasma. (2) Fresh serum, prepared by whatever method, has not been found to be toxic when precautions are taken to remove the fibrin filaments. Stored serum develops activity at the same time as stored plasma. (3) Whole blood contains more platelets than separated plasma. If Reid & Bick's view were correct, the plasma of whole blood should become toxic more rapidly, and should acquire a greater degree of toxicity than separated plasma. This, however, is not the case. Heparinized plasma stored with red cells, i.e. as whole blood, becomes toxic more slowly than separated plasma. There seems to be no difference in the degree of toxicity finally attained in the two cases.

The view that the toxic substance is a protein is supported by the fact that many other proteins and protein derivatives produce effects similar to plasma when injected into cats, and into some other animals. Pollitzer [1886] described a reaction in dogs and cats to the injection of Witte's peptone, and attributed the reaction to the presence in the 'peptone' of certain proteoses. Various other workers, including Chittenden, Mendel & Henderson [1899], Thompson [1896, 1899] and many more recent workers, have shown that the injection of peptones and proteins into cats and dogs produces vasodilatation with a fall in systemic arterial blood pressure. The mode of action of these substances has, however, remained obscure.

A number of authors have described a leucopenia in animals as a result of the intravenous injection of various substances. Bruce [1894] described the disappearance of leucocytes from the peripheral blood of rabbits after the injection of peptone. The polymorphonuclear leucocytes were affected, and their disappearance was not due to destruction, but to their accumulation in the blood vessels of certain organs, chiefly the lungs. There was also an increase in the polymorphonuclear leucocytes in the spleen and liver.

Widal, Abrami & Janovesco [1920] described a leucopenia in dogs, produced by intravenous injection of portal blood obtained during the digestion of proteins. They also noted a leucopenia in dogs in which an Eck fistula was made after a protein meal. They gave the name of 'haemoclastic crisis' to this reaction, which they attributed to the passage of 'albuminoses', products of protein digestion, which are normally removed by the liver, into the general circulation. These authors described a similar phenomenon in liver damage in man, and made this the basis of a test for liver function.

More recently, Staub & Butcher [1938] and Staub, Mezey & Golandas [1938] have described a leucopenia in response to injection of substances other than proteins. Solutions of glycogen, gum arabic, and starch produce this effect in dogs and rabbits. The granulocytes are more affected than the lymphocytes

and the cells which disappear from the blood are found in clumps in the lung vessels. They suggest that this is a result of the agglutination of leucocytes which have phagocytosed the injected macromolecules, and thus lost the power of passing through the lung capillaries. Vejlens [1938] noted a leucopenia as a result of the intravenous injection of various hydrophil colloids into guinea-pigs, rabbits and cats, and also as a result of decreased velocity of blood flow in these animals. He attributed the leucopenia to changes in the hydraulics of the circulation which caused the white blood cells to become 'marginal flowing' instead of flowing in the axial stream, and also made them adhere to the vessel walls. These changes in the distribution of white cells were considered by him to be part of the body's defence reaction to the injection of foreign substances.

In view of the similarities between the grosser symptoms of plasma injection and of anaphylactic shock, it is of interest to note that similar changes occur in the distribution of the blood cells in the two cases.

Biedl & Kraus [1909] described the occurrence of leucopenia in anaphylactic shock, and Andrewes [1910] noted this both in anaphylaxis in rabbits and as a result of injection of various bacteria intravenously in these animals. He noted, too, that polymorphs were present in excessive amounts in the liver.

In anaphylactic shock in dogs, Dean & Webb [1924] noted a polymorphonuclear leucopenia followed by leucocytosis. Webb [1924] subsequently showed that the leucopenia was due to polymorphonuclear cells adhering to vessels within the lung. Deep cuts in the lung substance, which produced marked haemorrhage, failed to release the adherent leucocytes. Dean [1922], in a case of anaphylaxis in man, reported that the sinusoids of the liver and vessels of the lungs were packed with white cells. Amongst the leucocytes were a large number of eosinophils. In addition he noted rupture of the alveolar walls in certain areas, but, as artificial respiration had been performed, this might possibly be ascribed to external trauma.

The injection of atropine very much decreases the cat's response to stored plasma. Auer [1910] showed that atropine diminishes anaphylactic shock in the guinea-pig. Lumière & Meyer [1933] showed that this was also true for the anaphylactoid response to injection of ground pumice stone. It seems possible, therefore, that anaphylactic shock, as well as the anaphylactoid responses to injections of stored plasma and serum, and of particulate matter, may in part consist of a reflexly evoked outburst of parasympathetic activity which is paralysed by atropine.

The results of the experiments indicate that the response to injection of stored plasma or serum is a reflex one. The reflex is stimulated by some constituent of the injected fluid and the efferent path is largely, but not entirely, via the parasympathetic nervous system. The muscular movements in the anaesthetized and decerebrate animals show that other parts of the nervous

system are involved. The receptors are mainly in the lungs, but, as the pupillary reactions to stored plasma in vagotomized animals show, part of the sensory pathway is from the abdominal area via the splanchnic nerves, possibly the pathway demonstrated by Bain et al. [1935] which they showed carried pupillo-dilator impulses from the intestines.

The speed with which the respiratory rhythm changes to that of the Cheyne-Stokes type is too quick to be produced by any oxygen lack or accumulation of CO_2. The records of intrapleural and pulmonary arterial pressures (Figs. 2, 5) suggest that the volume and pressure of blood in the lungs increases after the injection of stored plasma; whether this increased blood pressure and volume or the plasma itself stimulates the vagal lung receptors is uncertain. Partridge [1939] believes that the efferent vagal fibres which cause reflex acceleration of respiration in the dog are stimulated by circulatory changes in the lung. Hammouda, Samaan & Wilson [1943], however, deny this. Daly, Ludány, Todd & Verney [1937] showed that a rise in pulmonary arterial pressure may cause variations in respiratory rate; vagotomy abolishes this response. No effect is produced by making the animal breathe a fine cloud of plasma or by injecting plasma into the pleural cavity. If, therefore, the nerve endings in the lung are sensitive to a chemical substance, these endings must be in close apposition to the lung vascular system.

The 'immunity' to further injections of stored plasma mentioned above, which lasts for a period of about 35 min., is difficult to explain on any immunological basis. But, as the response is reflex in nature, the 'immunity' is doubtless due to a fatigue of sensory endings. This view is borne out by the effects of operative interference in the chest and overventilation by artificial respiration noted by Brodie [1900], and also by the fact, noted above, that the effects on respiration last for a period up to 20 min.

It is probable that the acute symptoms of massive pulmonary embolism in man are a manifestation of this reflex.

SUMMARY

1. Plasma or serum develops a toxicity on storing for 3–4 weeks. Stored human or cat plasma may kill a cat when so little as 0·5 c.c. is injected intravenously.

2. Clotting of plasma plays no part in the development of toxicity. Breakdown of platelets is not responsible, since filtered plasma becomes toxic at the same time as unfiltered plasma.

3. The activity is associated with a protein of the albumin class.

4. The effects of intravenous injection in the cat are: vagal inhibition of the heart, fall of blood pressure, altered respiration, increased peristalsis, micturition, defaecation, vomiting, constriction of the pupil, opisthotonos,

all of reflex origin, and a leucopenia which is followed, after some hours, by a leucocytosis.

5. The main sensory nerves for this massive reflex are the vagal lung fibres, and most of the effects are abolished by vagotomy or by atropine.

6. The mode of stimulation of the sensory nerve endings has not been determined, though it is possible that pressure changes in the pulmonary circulation may in some way act as a stimulus.

7. The changes in white blood cells, as well as the more gross responses to the injections, are very similar to those occurring in anaphylactic shock and in anaphylactoid conditions. These different conditions may merely represent different methods of eliciting a general physiological response to the injection of foreign matter into the circulation.

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REVERSIBILITY OF CARBOHYDRATE AND OTHER CHANGES IN RATS SHOCKED BY A CLAMPING TECHNIQUE

BY R. E. HAIST AND JEAN I. HAMILTON, *From the Department of Physiology, University of Toronto, Toronto, Canada*

Secondary shock may be produced by a variety of methods. In the experiments to be described, a simple clamping technique has been used to induce shock in rats. Employing this procedure it is possible to demonstrate that certain changes in the shocked animals may be reversed and the fatal outcome prevented. Liver glycogen studies indicate that during the period of shock that follows the removal of the clamps, the rats exhibit a reduced ability to store glycogen when glucose is administered. The ability to store glycogen in the liver returns when the injured limbs are reclamped.

METHODS

Female albino rats of the Wistar strain, weighing between 150 and 250 g., were used as the experimental animals. Each rat was suspended in a sling, the design of which was a slight modification of one previously used by Bunker & Solandt in this department. A clamp was applied to each hind leg of the rat. The clamps were made from rubber tubing clamps by the addition of curved metal plates (Fig. 1). The clamps were tightened sufficiently to cut off the circulation to the limbs. The nut on the clamp was tightened with a small, specially constructed, constant torque wrench. The clamps were retightened 15 min. after they were first applied. The clamps were left on overnight (12–15 hr.), though a shorter period may be used. When the clamps were removed, the limbs became swollen rather quickly and the animals died within a few hours. Throughout the test the rats were kept in a constant temperature box maintained at 27° C.

For all experiments in which glycogen estimations were made, food and water were removed 24 hr. prior to the estimated time of sacrifice. One c.c. 25 % glucose was given by stomach tube at the time the food was removed. The animals were anaesthetized with sodium amytal just before the liver samples were taken. The liver glycogen was determined by the method of

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Good, Kramer & Somogyi [1933]. Blood sugar levels were obtained by the Somogyi method [1937]. The absorption of glucose from the gastro-intestinal tract was estimated by the method of Cori [1925]. The limb volume was estimated by measuring the volume of fluid displaced from graduated cylinders
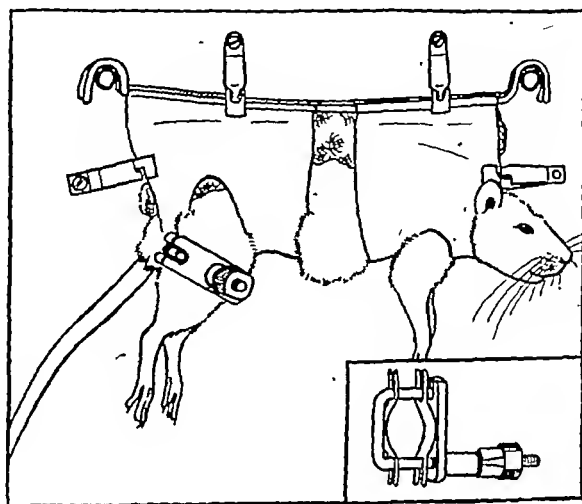
Fig. 1.

when the leg was immersed to a known level. The leg was first placed in water containing a wetting agent and each estimation was made in triplicate. The method, while relatively crude, gives values which are reproducible. This is shown by the results of three separate series of twelve successive single measurements:

Leg	Average volume c.c.	Standard deviation
1	12·3	±0·3
2	9·8	±0·4
3	8·3	±0·3

RESULTS

When the clamps are removed from the rats, the limbs swell and the animals die within a few hours. The survival times for 84 rats are shown in the histogram (Fig. 2). The average survival time is 3 hr. 12 min. and the mode lies between 2 and 2½ hr.

Liver glycogen in shock. In the shocked rats (clamps removed) low values for liver glycogen were observed (average, 0·043 g. % for 20 rats). The values are also low in the control rats fasted for 24 hr., though usually not as low as those in the shocked animals (average, 0·142 g. % for 20 rats). Animals which have the clamps left in position show intermediate values but are nearer the control levels (average, 0·085 g. %). The glycogen levels are illustrated in Fig. 3.

Survival times (S4 rats)
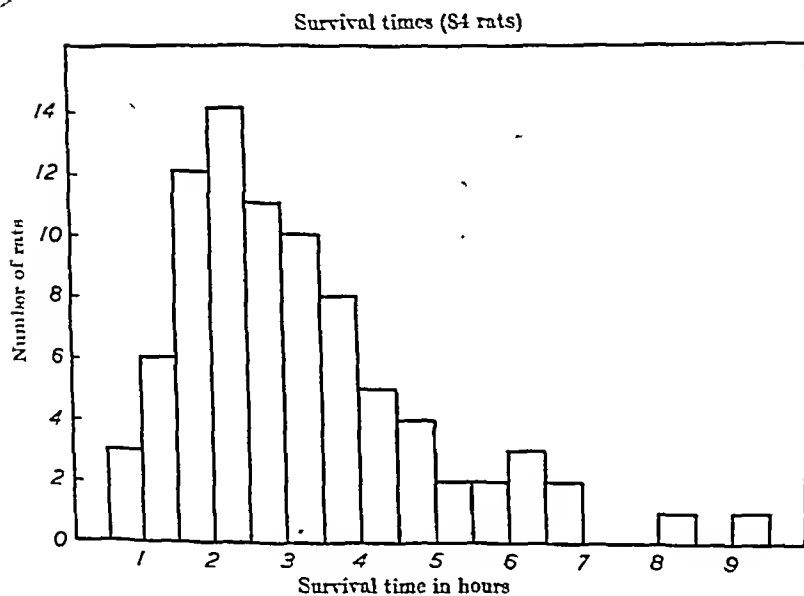
Fig. 2.
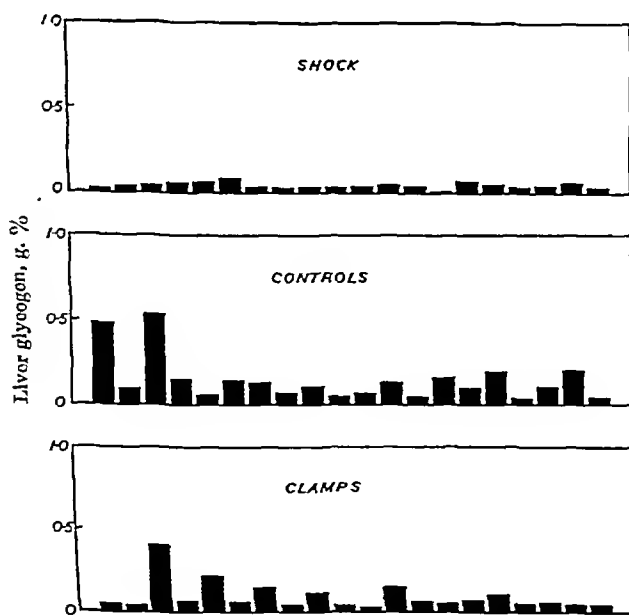
Fig. 3.

Glycogen storage following glucose administration. At the time the clamps were removed from the shocked rats, 2 c.c. 25 % glucose was given by stomach tube to each of the control and shocked animals. Liver glycogens were determined 4 hr. after the clamps were removed. Samples were obtained from control and shocked animals at the same time. Twenty control rats showed an
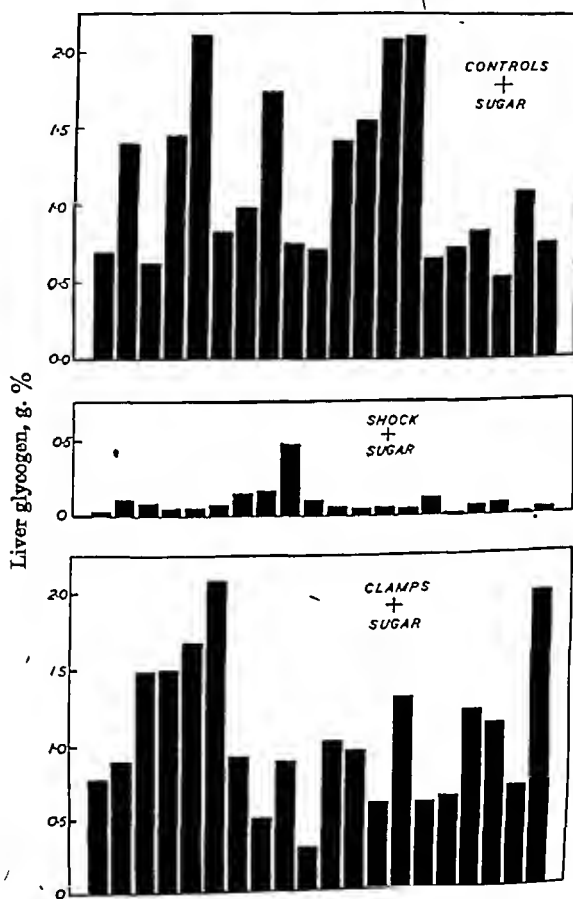
Fig. 4.

average liver glycogen value of 1·16 g. %, an increase of 1·0 g. %, or ten times the fasting value. Twenty shocked rats gave an average value of 0·093 g. %, an increase of 0·05 g. %. This increase is relatively slight, compared with that of the controls. The difference is not due to the clamping itself, since rats having the clamps left in place until after the liver was removed, responded like the controls when glucose was given. The average glycogen value for the clamped animals receiving glucose was 1·05 g. % or twelve times the level in

clamped rats receiving no glucose. The glycogen changes in the different groups following the administration of glucose are compared in Fig. 4. From this, chart it will be evident that the shocked rats exhibit an impaired ability to store glycogen in the liver when sugar is fed. The change occurs only after the removal of the clamps, that is, after the restoration of circulation to the legs, since the clamped rats store glycogen as do the unclamped controls.

Absorption of glucose from the gastro-intestinal tract. It was necessary to determine whether or not the impaired storage of glycogen in the shocked rats was due to a poor absorption of glucose from the gastro-intestinal tract. First, intraperitoneal injections of glucose were tried. The results were the same as when the glucose was given by stomach tube. Next, the absorption of glucose was compared in 10 shocked rats and in 10 clamped controls by determining the residue of reducing substances in the gastro-intestinal tract 4 hr. after the glucose was given by stomach tube. In both the shocked and control animals practically all the glucose had been absorbed at that time (Table 1).

TABLE 1. The absorption of glucose from the gastro-intestinal tract.
(2 c.c. 25 % glucose given by stomach tube. Rats killed in 4 hr.)

	Clamped controls				Shocked rats		
Rat wt. g.	Blood sugar mg. %	Intestinal residue. Reducing substances mg.	Glycogen g. %	Rat wt. g.	Blood sugar mg. %	Intestinal residue. Reducing substances mg.	Glycogen g. %
211	186	12	—	159	340	44	—
200	170	109	—	203	234	18	—
170	168	92	—	213	325	9	—
197	120	10	—	240	680	12	0·148
188	174	11	1·64	221	363	32	—
234	185	19	1·37	202	363	·31	0·436 ·
229	235	22	0·88	204	420	124	0·055
217	181	29	0·71	189	402	127	0·073
210	246	11	0·79	189	396	70	0·077
198	260	23	1·17	223	613	71	0·222
Average	192	34	1·09		414	54	0·168

While the actual rate of absorption is not measured in this experiment, it seems evident from the table that the impaired storage of liver glycogen in shocked rats is not due to poor absorption of sugar from the intestinal tract. The reduced storage of liver glycogen occurs despite the fact that the blood sugar level is very high in the shocked rats given glucose. The average blood sugar values for several different groups of rats are presented in Table 2.

It is clear that in the shocked animals there is a greatly diminished ability to store glycogen in the liver at a time when the blood sugar is very high. This suggests the possibility that the changes might be associated in some way with an insulin deficiency. The blood sugar levels in the clamped control rats were higher than in the fasting animals which were not clamped. The liver

TABLE 2. Blood sugar changes

Sample	No. of rats	Average blood sugar mg. %
Control rats (no clamps)	5	90
Control rats, 4 hr. after sugar	5	144
Clamped control rats	5	148
Clamped, 2 hr. after sugar	5	248
Clamped, 4 hr. after sugar	10	192
Shocked rats (clamps off)	5	76
Shocked, 2 hr. after sugar	5	406
Shocked, 4 hr. after sugar	10	414

glycogen values seemed to be slightly lower in the clamped group. This would seem to indicate that the clamping procedure itself has some effect on the breakdown of liver glycogen. However, as far as the storage following glucose administration is concerned, the clamped animals responded like the control rats.

The effect of insulin administration. In order to test the possibility that an insulin deficiency might be involved, 2 units of insulin were injected subcutaneously into shocked rats. Some difficulty was encountered because of the early death of many of the rats. The effect of insulin administration on liver glycogen is shown in Table 3. The data in Table 3 indicate that insulin injection does not increase the ability of the shocked rats to store glycogen in the liver.

TABLE 3. The effect of insulin administration in shocked rats

Shocked rats, each given 2 units of insulin subcutaneously		Clamped controls given no insulin, sacrificed at corresponding times	
Rat wt. g.	Liver glycogen g. %	Rat wt. g.	Liver glycogen g. %
194	0·244	165	0·82
212	0·061	200	0·20
197	0·035	180	1·23
189	0·061	220	0·26
195	0·071	220	0·43
160	0·008	176	1·42
176	0·063	210	0·80
182	0·041	180	1·36
193	0·000	186	1·24
200	0·037	196	0·68
219	0·014		
209	0·011		
189	0·045		
220	0·088		
Average	0·056*		0·84

* Average liver glycogen for shocked rats given glucose: 0·093 g. %.

Table 4 shows the average blood sugar levels for the different groups at corresponding times after the glucose was given by stomach tube. From this it would appear that in the shocked rats the injection of insulin results in a very definite reduction in the blood sugar level, even though it does not enhance the storage of liver glycogen.

TABLE 4. Average blood sugar levels at corresponding times after sugar administration.
(500 mg. glucose/rat)

Group	No. of rats	Average blood sugar mg. %
Clamped controls	5	248
Shock	5	406
Shock-insulin	13	134

The effect of oxygen lack on glycogen storage. Because of the prevalent opinion concerning the importance of fluid loss and the resultant development of tissue anoxia, it seemed desirable to determine whether or not oxygen lack, in itself, would alter the ability of the animal to store glycogen in a manner comparable to that observed in shock. Experiments were conducted at different atmospheric pressures in a special chamber. Each rat was given 2 c.c. 25 % glucose solution by stomach tube and the liver glycogen was determined at the end of 4 hr., as in the shock experiments. Immediately after the glucose was given, the animals were put in the chamber and the pressure was reduced. The low pressure was maintained throughout the 4 hr. period. The results are shown

TABLE 5. Glycogen formation in low oxygen atmospheres
Liver glycogens in g. % 4 hr. after glucose administration at approximate altitudes of
16,000 ft. (409 mm. Hg), 25,500 ft. (275 mm. Hg), and 30,500 ft. 220 mm. Hg)

	Liver glycogen, g. %	
409 mm. Hg	275 mm. Hg	220 mm. Hg
0·96	0·487	0·487
0·98	0·161	0·107
1·27	1·180	0·123
0·65	0·113	0·091
1·26	1·220	Died
—	—	0·074
—	—	0·027
—	—	0·066
—	—	0·109
—	—	Died
Average 1·02	0·632	0·135

n Table 5. From this table it will be seen that at 409 mm. Hg (16,000 ft.) the animals stored glycogen in normal amounts. At 275 mm. Hg (25,500 ft.) some rats showed diminished storage but some did not. At 220 mm. Hg (30,500 ft.) two of the animals died and with one exception the others showed reduced ability to store glycogen. When the oxygen want is severe the diminished storage of liver glycogen approaches that observed in shock, but of course the conditions are not necessarily comparable, as will be considered later.

Reversibility of shock

The effect of reclamping on survival. In the experiments already outlined, shock was produced when the circulation was restored to limbs that had been clamped for 12–15 hr. After removal of the clamps the limb volume increased

rather quickly, reaching a maximum within 2 hr. from the time that the clamps were taken off. At 1 hr. after removal the major part of the swelling had occurred. These facts are evident in Fig. 5, which shows the increase in volume of the hind limbs with time.

It has been found that if the limbs are reclamped at a time when the swelling is complete, or nearly so, most of the animals will survive. The clamps used at this time are very narrow and are placed as high on the legs as possible. They
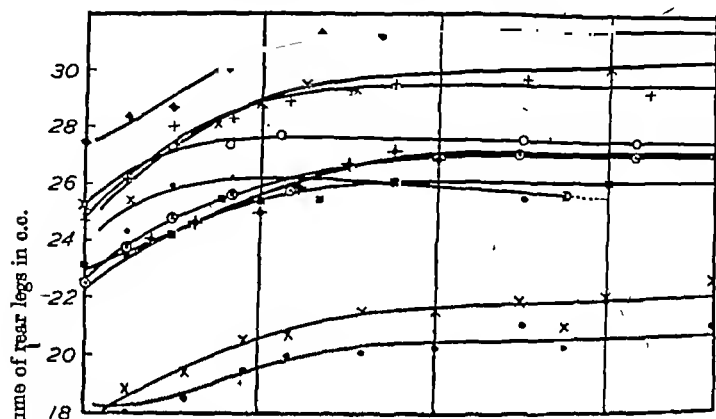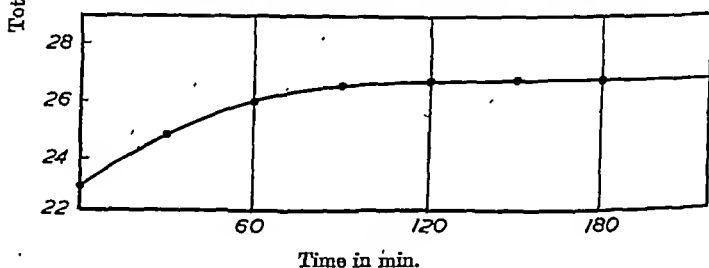
Fig. 5.

are tightened sufficiently to stop the circulation to the limbs. Limb volume measurements taken many hours after the reapplication of the clamps indicate that there is no significant reduction in limb volume after reclamping. The fluid trapped in the injured limbs is lost to the body as a whole.

In the shocked rats before death the respirations become laboured and finally gasping in type. Breathing stops several minutes before the heart. Reclamping after the respirations become laboured seems to be less effective. Animals reclamped at that time usually die within the first hour after the clamps are reapplied. Some of the reclamped animals used in glycogen and

sugar studies were killed 4 hr. after reclamping. They were in good condition at that time. All the others were killed, in good condition, more than 7 hr. following reclamping. Thirty-five were kept for more than 20 hr. before they were killed. Twenty-four shocked rats, reclamped at 2 hr., were kept for 48 hr. before they were killed. None had received food since 24 hr. prior to release of the clamps. Some of the data concerning the reclamped animals are given in Table 6. It is evident from the table that reclamping the injured limbs encourages survival despite the fact that the local fluid loss appears to be practically complete at the time that the clamps are reapplied, and the loss is maintained by the reclamping.

TABLE 6. Reclamping and survival

No. of rats shocked	Time after removal when clamps reapplied hr.	No. dying before reclamped	No. reclamped	No. dying in 30 min. after reclamping. Almost dead before	Survivals
61	1	13	48	5	41
78	2	18	60	7	40

The effect of reclamping on the storage of liver glycogen. It was important to determine whether there was a restoration of the ability to store liver glycogen when the clamps were reapplied. Experiments were performed on rats in which the clamps were reapplied 1 hr. and 2 hr. after removal. Each rat received 2 c.c. 25 % glucose solution by stomach tube immediately after the clamps were reapplied. Glycogen determinations were made 4 hr. after the glucose was given. The results are shown in Table 7.

TABLE 7. The effect of reclamping on glycogen storage in the livers of shocked rats.
(Sugar was given at the time of reclamping. Glycogen was estimated 4 hr. later)

	Liver glycogen g. %		Blood sugar mg. %	
	Reclamped after 1 hr.	Reclamped after 2 hr.	Reclamped after 1 hr.	Reclamped after 2 hr.
	1·87	0·43	430	382
	1·09	0·37	256	336
	1·48	0 46	240	388
	0·95	0·59	343	421
	0·18	0 63	239	255
	0·38	0·37	204	180
	0·29	0·19	228	253
	1·18	0·94	—	—
	0·59	0·34	—	—
	0·12	0·30	—	—
	0 45	—	—	—
Average	0·78	0·46	277	316

Average liver glycogen for shocked rats given sugar: 0·093 g. %.

The ability of the rat to store liver glycogen returns towards normal when the injured legs are reclamped. It would seem that this restoration may be more rapid when the clamps are reapplied earlier in shock. Since the animals survive, it is likely that other abnormalities in the shocked rats also return

to normal. It is well to point out, however, that the return in the ability to store glycogen in the liver takes place very quickly.

A comparison of liver glycogen and blood sugar values for all groups. A comparison of the average values for liver glycogen and blood sugar in the different groups is given in Fig. 6.

Average liver glycogen and blood sugar values
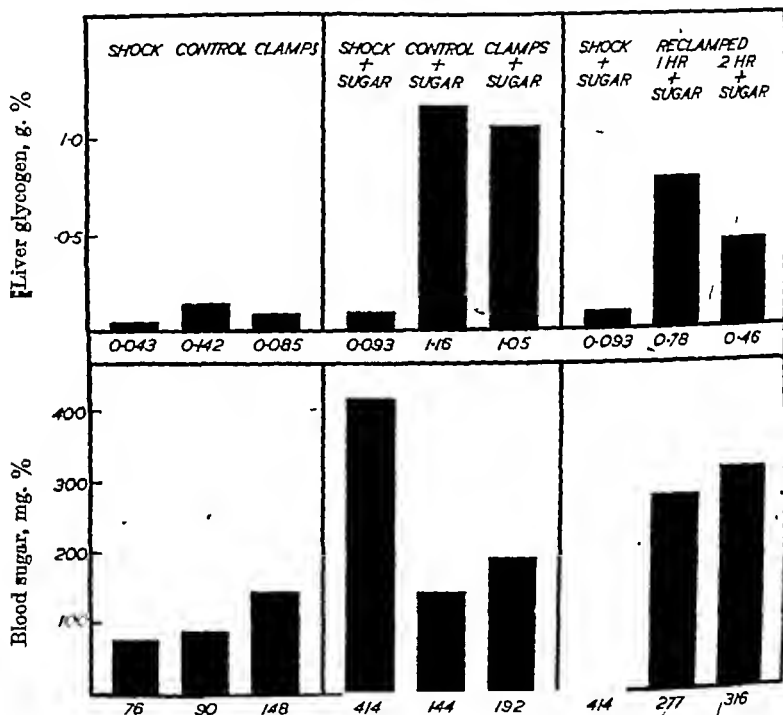
Fig. 6.

DISCUSSION

A method of producing shock in rats has been described. Rats shocked by this procedure exhibit an increase in limb volume. Most of the increase appears in the part distal to the clamped region. The degree of swelling and the rapidity with which it occurs probably indicate the extent of the restoration of circulation to the limbs. If the swelling is rapid and extensive the animal is more apt to die quickly. If the swelling is slight the animal is likely to survive for a longer time. It is reasonable to suppose that the major factor in shock must be associated with the limb swelling in some way. At first thought this might suggest that the local fluid loss itself is the important factor in causing shock. Since the reapplication of clamps to the damaged limbs after they have swollen does not seem to diminish appreciably the local fluid loss, and since the

reclamped rats survive, it seems unlikely that local fluid loss itself is the fundamental factor in this type of shock. The possibility that fluid spreading above the clamped area or that loss of fluid from the surface of the legs may become important factors after the local swelling is complete, must be considered. Since the clamps are reapplied relatively close to death, the importance of these factors becomes questionable. The fact that the limb swelling is maintained after reclamping argues against any significant influence of fluid loss from the surface of the legs.

The information in the literature concerning glycogen changes in shock is not very extensive. Reduced values for liver glycogen have been reported following haemorrhage [Aggazzotti, 1927]. In our experiments, the fasting shocked rats (clamps removed) showed a reduction in liver glycogen below that of control animals with or without the clamps. Despite this, the blood sugar in the shocked animals was lower than in the control groups, indicating either (1) that there was an increased peripheral utilization of glucose, or (2) that there was a diminished production of glycogen from non-sugar sources in the shocked rats. Engel, Winton & Long [1943] mention some unpublished work of Russell & Engel on eviscerated rats which indicates that there may be an increased rate of glucose utilization by peripheral tissues in the shock following haemorrhage.

When the shocked rats are given sugar by stomach tube it is found that they exhibit a diminished ability to store glycogen in the liver. The clamped control animals store glycogen essentially as well as the unclamped controls. Hence, the clamping, in itself, is not responsible for the changed function. The absorption experiments show that the diminished ability to store liver glycogen is not due to poor absorption of glucose from the gastro-intestinal tract. Also the poor storage occurs despite the fact that the blood sugar level is very high. Insulin administration lowers the blood sugar level in the shocked animals but does not seem to help the animal to form glycogen from ingested sugar. The evidence seems to indicate that the poor storage of liver glycogen in shock is probably not due to a deficiency of insulin. However, since injected insulin will lower the blood sugar in the shocked rat and since the blood sugar in shocked animals receiving glucose is maintained at a high level for long periods, it follows that either insulin secretion is not regulated by the blood sugar level or that such a mechanism is not functioning adequately in shock.

The experiments concerning the effect of reduced atmospheric pressure show that when oxygen lack is extensive there is a diminished storage of liver glycogen after glucose administration. The diminished storage approaches that observed in shock. Evans [1934] reported that low atmospheric pressures lead to an increase in liver glycogen. However, the conditions for the experiments reported here are different in that the exposure was for a much shorter time and the atmospheric pressure was lower. It is realized that a condition of

generalized anoxia of this type probably is not comparable to the condition in the shocked animals since in shock the cardio-vascular compensations tend to maintain an adequate supply of oxygen to the brain, and the anoxia where it exists is due to a reduced blood flow through the tissues rather than to poor oxygenation of the blood.

It is desirable to know whether the changes in the shocked animals are due to a local oxygen deficiency or whether they are brought about by a secondary more generalized anoxia. When the clamps are reapplied to the legs and the damaged (and anoxic) tissue is cut off from the rest of the body, most animals survive and the ability to form liver glycogen is restored. The local loss of fluid is maintained by the reclamping, hence if a generalized anoxia following the local change is the important factor it is difficult to see why this factor ceases to operate in the reclamped animals. If there is a generalized anoxia that is not the result of a reduction in blood volume following the local fluid loss but the result of an altered circulation due to other causes, then reclamping might bring about its effect by restoring the circulation.

It is evident from these experiments that some change occurs in the damaged (and anoxic) tissue which affects the activity of tissues in other parts of the body remote from the site of injury. This influence can be removed by reclamping or blocking off the injured part from the rest of the body. The nature of the process by which the effect on more remote tissues is brought about has not been elucidated as yet.

One important point emerging from the reclamping experiments is that the profound changes in the shocked animals are reversible until late in shock, even at a time when transfusion is of little use. It is probable that the important irreversible changes, if such there be, occur just prior to death. The hope is raised that, by reversing some of the earlier changes, shock may be alleviated or prevented. Obviously, reclamping is not a practical means of achieving this and some more feasible procedure must be found.

SUMMARY

1. A method of producing shock in rats by the application of clamps to the limbs is outlined.

2. The average survival time of 84 rats shocked by this procedure is 3 hr. 12 min.

3. The liver glycogen values are somewhat lower in the shocked rats than in clamped control animals or in unclamped controls.

4. Following the administration of sugar by stomach tube, the shocked rats are unable to store glycogen in a normal fashion. This occurs despite the fact that the blood sugar level is very high in the shocked rats and the absorption of sugar from the gastro-intestinal tract is almost as complete as in the clamped control animals.

5. Insulin administration does not improve the glycogen storage in the liver, but lowers the blood sugar level in the shocked rats.

6. With severe oxygen want there is a diminution in the storage of liver glycogen after glucose administration approaching that observed in shock.

7. When the shocked animals are reclamped before they are on the point of death and after the local fluid loss is practically complete, most of them survive. This happens despite the fact that the fluid trapped in the limbs is lost to the body.

8. When the shocked animals are reclamped, they rapidly recover the ability to store glycogen in the liver when glucose is administered.

9. It is concluded that local fluid loss is not the fundamental factor in shock in these animals.

10. Some change occurs in the damaged (anoxic) tissues which affects the activity of tissues in other parts of the body. How this effect is mediated has not been determined.

11. Extensive changes in the shocked animals are reversible until late in shock.

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ABSORPTION OF SERUM PROTEINS FROM THE INTESTINE OF THE SENSITIZED GUINEA-PIG

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By use of the anaphylaxis reaction, it has been shown [Winter, 1944] that horse serum, albumin, and globulin, injected into the duodenum of appropriately sensitized guinea-pigs, are absorbed, and bring about desensitization of the uterus. This effect was observed only when the proteins were introduced at or above a certain minimal concentration. The amount of serum required to desensitize, especially in the serum-sensitized animal, was so large as to suggest that distension of the gut was a factor which might play a part in absorption. This suggestion had previously been made by Hettwer & Kriz [1925], who showed that absorption of serum from the small intestine of the serum-sensitized guinea-pig was sufficiently rapid to cause anaphylactic shock, if the intestine was markedly distended by the injected fluid. In our experiments no symptoms of shock were observed, but it was possible that the temporary distension of the duodenum, which would persist until the serum was distributed along the gut, might last long enough to induce absorption of sufficient protein to cause desensitization of the uterus, while the speed of absorption was too slow to cause shock. Beyond the fact that $3\frac{1}{2}$–4 hr. was sufficient, no information as to the time needed to desensitize the animals was obtained in the previous work, which was concerned with the minimum amounts of serum, albumin, and globulin necessary to cause desensitization when introduced into the intestine. The object of the experiments about to be described was to determine whether the three different proteins might desensitize the animals in a shorter time; if absorption of serum is appreciably dependent upon distension of the intestine, which, under the conditions of these experiments, can only last a matter of minutes, then serum should be more effective than the other proteins at the minimum time allowed.

RESULTS

Desensitization by proteins injected into the duodenum

Milk-test guinea-pigs were used; their average weight was 373 g. The propriety of using these animals has previously been discussed [Winter, 1944]. The method

of sensitization and the preparation of the proteins used were similar to those described in the previous paper, with the exception that the serum animals were sensitized only for 15–20 days. In all the experiments in which serum proteins have been introduced into the duodenum, the site of injection was, as before, about 4 cm. from the junction with the pylorus, and the amount of protein was the minimum which had previously been found necessary to effect desensitization 3½–4 hr. after the injection. In the present series of experiments the proteins were injected 3–4 hr. after the first uterine horn had been removed for test of sensitivity, which was determined in all cases by the addition of 0·1 c.c. of whole serum to the bath. Groups of ten animals were used. In the first series, the guinea-pigs were killed 15 min. after injection. The results are given in the table, and are those which had been expected, namely, that the largest number of animals are desensitized by serum, probably due to distension of the gut; a smaller number are desensitized by globulin than by albumin, which has the smaller molecule. In the expectation that, with a longer interval, more animals would be desensitized by globulin and by albumin than in the first series, while the serum results would be substantially the same, a second series of experiments was undertaken; this was identical with the first, except that the animals were killed ½ hr. after the injection of the protein into the gut.

TABLE 1. Injection of serum proteins into the duodenum

Sensitization	Protein injected	Amount injected per 100 g. body wt.	Number of animals desensitized	
			After 15 min.	After 30 min.
Globulin	Globulin	6·0 mg.	5	8
Albumin	Albumin	6·0 mg.	7	7
Serum	Serum	0·95 c.c.	9	5

From a consideration of the above two series, we may conclude that the rate of absorption of globulin from the intestine is such that, after 15 min., only 50 % of the animals are desensitized, and 80 % after 30 min. In the absorption of albumin, there is no difference between the two series, 70 % of the animals being desensitized in each case. The serum experiments however showed, surprisingly, that a smaller number of animals were desensitized in half an hour, compared with those in which only 15 min. were available for absorption to take place. The difference was considered to be outside the experimental error of the method, but it seemed advisable to test this peculiar effect of the absorption of serum by carrying out two series of experiments in which serum was injected into the duodenum of animals sensitized by globulin. Ten animals were used for each series, and they were killed 15 and 30 min. after injection; the dose was 0·66 c.c. serum per 100 g., the minimum necessary for desensitization in this type of animal. Nine guinea-pigs were desensitized after 15 min., seven after 30 min. The results were thus identical in the 15 min. series, and

confirmatory, though not in quite such a striking manner, in the 30 min. period, of those obtained in the absorption of serum from the gut of the serum-sensitized animal.

Desensitization of serum-sensitized animals by serum injected into the circulation

The experiments in which serum was injected into the duodenum of serum-sensitized and of globulin-sensitized guinea-pigs suggested that a number of these animals had regained sensitivity after the uterus had been fully de-sensitized, i.e., that dissociation of the serum-antibody combination had occurred in some cases. It seemed possible that this might be correlated with the inexplicable failure, in the previous work, to obtain regular desensitization, $1\frac{1}{2}$ hr. after injection, of the serum-sensitized and globulin-sensitized animals by serum injected into the portal blood, irrespective of the amount of serum given [Winter, 1944, Tables 10 and 11]. No such inconsistency was observed when animals sensitized by serum were desensitized by globulin injected into the portal blood, nor when globulin-sensitized animals were given 'synthetic serum' by the same route [Winter, 1944, Tables 8 and 14]. It was argued that, if the results in Table 1 obtained from the serum-sensitized and globulin-sensitized animals and those previously reported were valid, they would imply that serum given into the portal blood would be capable of regular desensitiza-tion of serum-sensitized and globulin-sensitized animals when the animals were killed 15 min. later. The failure of serum to act uniformly as a desensitizing antigen after a longer period might then be due, not to fixation of the antigen by sensitized cells in the liver, but to temporary dissociation of the serum-antibody complex. This point was tested by injecting a series of serum-sensitized guinea-pigs with a very small dose of serum (0·01 c.c.), given into the mesenteric vein, to effect some preliminary desensitization and to avoid death in shock. The first uterine horn was removed and tested; 3–4 hr. later, the sensitive animals received a further injection of serum into the portal blood, the moderate dose of 0·02 c.c./100 g. being chosen. Ten sensitive animals were used; they were killed 15 min. after the second injection; all were desensitized. These results agree with those in which the serum-sensitized animals were de-sensitized by serum absorbed from the duodenum in 15 min., with the excep-tion of one in each series of ten. Entirely uniform results cannot be expected in experiments involving absorption from the intestine, owing to the number of factors which cannot be controlled, and it seems permissible to conclude that serum, whether introduced into the circulation directly, or after absorption from the intestine, desensitizes the serum-sensitized animal rapidly; and that there follows, in some cases, a period of renewed sensitivity. Presumably, de-sensitization is re-established $3\frac{1}{2}$–4 hr. later, otherwise it would have been im-possible to obtain the consistent results which were found, after this period,

when the minimum level of serum necessary to desensitize the serum-sensitized animal from the intestine was being determined [Winter, 1944, Table 20]. This was, however, confirmed by seven experiments on animals sensitized by serum, in which, after a preliminary injection of 0·01 c.c. serum into the portal blood, 0·02 c.c./100 g. were given by the same route. The animals were killed 3½ hr. later, and all were found to be insensitive.

Desensitization of globulin-sensitized animals by serum injected into the circulation

It was now necessary to determine whether the failure to effect, in all cases, desensitization of the globulin-sensitized guinea-pig by serum injected into the portal blood, though the dose of serum varied between 0·0075 and 0·10 c.c./100 g. body weight, might be explained on the same lines as in the case of the serum-sensitized animal. A number of animals sensitized by globulin received a preliminary injection of 0·01 c.c. serum into the portal blood, after removal of the first uterine horn. A further injection of serum (0·02 c.c./100 g.) into the portal blood was given 3–4 hr. later to ten sensitive animals, which were killed after an interval of 15 min.; the second uterine horns of three were sensitive. It appears, therefore, that globulin-sensitized animals cannot always be de-sensitized by serum injected into the portal circulation, whether the interval between injection and killing the animals is ½ hr., as in the present experiments, or 1½ hr., as in the previous work. This finding suggested that dissociation of the complex formed by interaction of serum and globulin-antibody might not be an adequate explanation in the globulin-sensitized animals, unless similar results could be obtained after injection of serum into the systemic circulation. Serum given by this route should be, on occasion, ineffective, if dissociation of the antigen-antibody complex took place. It should, however, always be effective if the failure to desensitize, when given into the portal blood, was due to fixation of globulin by sensitized cells in the liver. To test this point, guinea-pigs received an injection of 0·01 c.c. serum into the inferior vena cava, as being the most convenient site when the abdomen was open after removal of the first uterine horn. After the usual interval, twenty sensitive animals were again anaesthetized with ether and these received, via the jugular vein, the usual dose of serum (0·02 c.c./100 g.). Ten of the animals were killed after 15 min., and ten after 30 min.; the uterus was insensitive in every case. Serum is therefore uniformly capable, at this level, of effecting desensitization of the globulin-sensitized animal, and we must ascribe the failure to desensitize, when given into the portal blood, to some fixation of serum by cells in the liver. This fixation of serum by the liver seems to take place in a capricious manner in some experiments only, and no explanation can at present be put forward.

The occurrence of shock in globulin-sensitized animals

A distinct difference has been noted in the susceptibility to shock of the globulin-sensitized guinea-pigs, compared with those sensitized by serum for the same period. In the series of serum-sensitized guinea-pigs given an injection of 0·01 c.c. serum into the portal blood, followed by 0·02 c.c./100 g., there were no symptoms of shock in any animal. In the corresponding series of globulin-sensitized animals, three showed moderately severe shock after the first, and one after the second injection of serum; all, however, recovered. Of the twenty globulin-sensitized animals which received the preliminary injection of serum into the inferior vena cava, and the main dose into the jugular vein, five reacted with shock after the first injection and one after the second. There appears to be a greater tendency to shock following injection of serum in the globulin-sensitized animal, as compared with the animal which has been sensitized by serum. The same thing was observed, when, for another purpose, it was necessary to give serum into the portal circulation of serum-sensitized and globulin-sensitized guinea-pigs in a dose of 0·02 c.c./100 g., without the usual small preparatory injection of 0·01 c.c. These were virgin animals, which had not been injected with milk. The numbers were small, four serum- and five globulin-sensitized animals, but none of the former showed any symptoms, while two of the latter died in shock 4 min. after injection. The stage of sensitization was the same in each group, viz. 3 weeks. It is not intended to suggest that this dose of serum can regularly be given to serum-sensitized animals without causing shock; in fact, Table 11 of the previous paper shows that two serum-sensitized guinea-pigs died in shock after an injection of serum less than 0·02 c.c./100 g., but the findings in the short series in which this dose was given to the two groups of animals agree with those described above.

DISCUSSION

It has already been shown [Winter, 1944] that a large volume of serum (1·5–2·8 c.c.) must be introduced into the intestine of sensitized animals in order to render the uterus insensitive, whereas relatively small amounts are required to desensitize when serum is injected directly into the blood. This fact, together with the rapid desensitization which follows the introduction of serum into the intestine, makes it probable that an initial small amount of serum rapidly passes through the intestinal wall, on account of distension of the gut, and it is this part which desensitizes the animal, the remainder not being absorbed, or only after some action by the digestive enzymes. If this view is correct, it is scarcely possible that serum can be absorbed except under the artificial conditions which cause intestinal distension. It is otherwise in the case of albumin and globulin, where these proteins were injected into the duodenum in a volume of fluid which did not exceed 1·0 c.c.; appreciable amounts must pass from intestinal lumen to blood in 15–30 min.

It is still undecided whether the rate of absorption of albumin from the small intestine is greater than that of globulin, but it is perhaps worthy of note that only in the case of globulin, which may be carried by the lymphatic system after absorption [Winter, 1944], was there found the expected difference between the animals killed 15 and 30 min. after injection of the protein into the duodenum. Too much reliance, however, must not be placed on small differences, when the proteins are being absorbed from the intestine, since it is impossible to control certain factors, such as the activity of the digestive enzymes and the passage of stomach contents into the duodenum. All the animals were fed as usual in the previous afternoon, since it was important that they should be in the best possible condition to withstand the operative procedure. There is a discrepancy, however, in the results, which cannot be accounted for on these lines. Three out of the ten globulin-sensitized animals were still sensitive after the introduction of serum directly into the portal circulation, while only one was sensitive when the serum was absorbed from the intestine, the animals being killed 15 min. later: the latter series should have yielded as many, or more, sensitive animals than the series in which the protein was injected into the portal blood. If serum, after leaving the intestine, were carried by the lymphatic system, instead of by the portal blood directly to the liver, the discrepancy would disappear. No failure to desensitize the uterus occurred when the serum was injected into the systemic circulation: on the other hand, it has been shown that certain cells in the liver of the globulin-sensitized animal bring about fixation of serum in some cases. This explanation is, however, put forward with all reserve, until more direct evidence is available.

The results in the series of serum-sensitized animals, given serum into the duodenum and killed 15 and 30 min. later, are difficult to understand, unless some animals, which were desensitized in the shorter period, had regained sensitivity. This conclusion is necessary, since serum will regularly desensitize serum-sensitized animals when the antigen is injected into the portal circulation, and allowed to act for 15 min., while it was, on occasion, ineffective after an interval of $1\frac{1}{2}$ hr. Serum again became effective when it had been in the tissues for $3\frac{1}{2}$–4 hr. It seems possible that dissociation of the combination between serum and serum-antibody may take place at some time shortly following the injection of the protein into a serum-sensitive guinea-pig. Indications of this have been noted only in some animals, and since the combination of antigen and antibody is usually regarded as stable, at any rate for a much longer period, this suggestion can only be made tentatively, to explain the findings in the present work.

SUMMARY

1. Horse albumin, globulin, and serum, in the minimal amounts required to desensitize the uterus, have been injected into the duodenum of guinea-pigs sensitized by the corresponding protein. The animals were killed 15 and 30 min. later, when the uteri were examined for sensitivity.

2. The results obtained with serum suggest that distension of the intestinal wall may be a significant factor in the mechanism of absorption of this protein.

3. With the longer time for absorption, globulin alone gave rise to a greater number of desensitized animals.

4. Anomalous results were obtained when serum was absorbed from the duodenum of serum-sensitized and of globulin-sensitized animals. These results are discussed.

5. Animals sensitized by globulin show a greater tendency than serum-sensitized guinea-pigs, at the same stage of sensitization, to develop anaphylactic shock as a result of the injection of equal amounts of serum.

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THE EXCRETION OF DIODONE BY THE ISOLATED PERFUSED DOG KIDNEY

By A. HEMINGWAY and A. SCHWEITZER, *From the Department of Physiology. School of Medicine, Leeds*

The claim that the renal plasma flow (R.P.F.) is identical with the plasma clearance (C_D) of the diethanolamine salt of 3-5-diiodo-4-pyridone-N-acetic acid (Diodone (B.P.C.)) rests on the assumption that at low plasma concentrations [e.g. 0·8–2·0 mg. I/100 ml.] plasma is completely cleared of the substance during one passage through the kidney and that the blood corpuscles make no contribution. There is evidence, however, that this assumption is an oversimplification of the processes occurring in the kidney and that, in fact, two factors operate in opposing directions to prevent C_D being identical with R.P.F. These problems have been investigated by White [1940], White & Heinbecker [1940] and Corcoran, Page & Smith [1941] in experiments on the subcutaneously explanted dog kidney. They have shown, on the one hand, that the over-all extraction of the plasma is incomplete and, according to the extent to which this occurs, C_D is less than R.P.F.: on the other hand, these workers suggest that the red blood corpuscles contribute diodone to the plasma as the substance is removed from it and this tends to make C_D greater than R.P.F. The incomplete extraction of the plasma is, however, not entirely balanced by the contribution from the cells, and C_D, as measured, is less than R.P.F. In their experiments, R.P.F. was calculated from measurements of plasma clearances and renal arterio-venous differences for inulin, and compared with C_D. Corcoran *et al.* found R.P.F. = 1·15 C_D, and White & Heinbecker R.P.F. = 1·2 C_D. In the work about to be described, renal plasma flow has been measured directly in the isolated perfused dog kidney and compared with C_D. Comparisons, additional to those already known [Starling & Verney, 1922; Hemingway, 1935; Shannon & Winton, 1940], have been made between the behaviour of the isolated and the intact canine kidney.

METHODS

A pump-lung-kidney preparation was made in the customary fashion. Blood flow was measured by collecting the venous outflow, and R.P.F. was calculated from this after determination of the haematocrit value.

Estimations of organic iodine in plasma and urine were made by the improved method of White & Rolf [1940]. By this method organic iodine in aqueous solution was estimated with an accuracy of $\pm 2\%$ within a concentration range of 1–2 mg. I/100 ml. In accordance with the findings of White & Rolf, the recovery of added iodine to plasma was only 80–85%, but since the percentage recovery, in our experience, varied slightly with different samples of plasma a 'recovery factor' was determined for the plasma used in each experiment and applied as a correction to the estimations made for that experiment.

Samples of arterial blood for determination of plasma I were withdrawn slowly from the tube leading to the perfusion cannula at the middle of each period of urine collection and were centrifuged immediately to minimize the effect of diffusion of diodone from cells to plasma. Urine samples were diluted to approximately similar concentrations as plasma before estimation.

Plasma iodine concentrations were maintained in each experiment at a level of approximately 1–2 mg. organic I/100 ml. plasma, after giving a priming dose equivalent to 1 mg./100 ml. plasma, by a steady infusion of a solution of diodone 400 mg./100 ml. 0.9% saline at a rate adjusted according to the rate of blood flow and previous experience of the likely degree of plasma extraction.

RESULTS

After the addition of diodone to the circulating blood and the starting of the infusion it is usually 10–15 min. before the concentration in the urine, when the urine flow is constant or increasing slowly, reaches an approximately steady level. This contrasts with the elimination of substances such as inulin or ferrocyanide which reach maximum concentration in the urine within 2–3 min., particularly with high rates of urine flow.

Comparison of R.P.F. and C_D. Twenty observations in the course of experiments where the kidney has been perfused at mean pressures of 110–120 mm. Hg have shown C_D to be less than R.P.F. The mean ratio of plasma C_D to R.P.F. with plasma levels of 1–2.5 mg. I/100 ml. is 0.58 (range 0.26–0.97).

Plasma extraction ratio of diodone. The mean plasma extraction ratio,

$$\frac{\text{art. plasma conc.} - \text{venous plasma conc.}}{\text{art. plasma conc.}}$$

$[E_p]$, is 0.62, with extremes of 0.23 and 0.87, in twenty observations. Disregarding one experiment in which all the extraction ratios were anomalously low (0.43–0.23), the mean plasma extraction ratio was 0.73 (range 0.62–0.87).

Both White & Corcoran and his co-workers refer similarly to unusually low values for E_p which they occasionally obtained from the explanted kidney.

E_p invariably falls during the course of an experiment even though the plasma concentration and the rate of blood flow remain unaltered. Thus in three consecutive 10 min. periods in one experiment in which the plasma I concentration was 1·65 mg./100 ml. and the blood flow was 95–100 ml./min., E_p was 0·87, 0·76, 0·67. Within the range of plasma concentrations employed in the present experiments the gradual fall in E_p does not appear to be related to any gradual changes in blood flow which occur during the course of an experiment (the perfusion pressure being constant), or to small alterations in the arterial plasma I concentration or the renal plasma load (R.P.F. × plasma concentration). Both gradually rising and falling plasma loads have been observed in particular experiments, but there is always a diminishing E_p, as the experiment proceeds.

But if the renal plasma load is suddenly altered greatly, as by changing the rate of blood flow, then apparently related changes in E_p are observed. The effect on E_p of lowering the perfusion pressure and reducing the renal blood flow for a period was observed in an experiment, the results of which are given in Table 1.

TABLE 1

Period	Perfusion pressure mm. Hg	R.P.F. ml./min.	Renal plasma load mg. I/min.	E_p
1	120	97·5	0·96	0·70
2	90	58·0	0·79	0·80
3	120	85·3	0·85	0·71

During the period of reduced blood flow and renal load E_p is elevated.

Passage of diodone between plasma and cells. The 'apparent' R.P.F. can be calculated from C_D/E_p. But if the calculated value is compared with the 'true' R.P.F., as determined from the measured blood flow, the 'apparent' R.P.F. is less than the 'true' R.P.F. and in the mean = 0·94 R.P.F. The deviation from unity, if interpreted literally, would mean that diodone is passing from plasma to cells during the period of a renal passage. Although this is just possible because the diodone infusion was made into the main blood reservoir from which blood was drawn for the perfusion of the kidney and a slight diffusion of diodone from plasma to cells might have continued after the point at which the arterial blood sample was taken, the discrepancy is more likely to be the result of experimental errors. Since the major part of the blood in the pump-lung-kidney preparation may be regarded as *in vitro*, it seems likely, from the observations of Smith, Goldring & Chasis [1938], that the diffusion of diodone between cells and plasma will occur slowly, and because the 'apparent' R.P.F. is not greater than the measured R.P.F. it is assumed that in the pump-lung-kidney preparation the contribution of diodone from the cells to the plasma is negligible.

DISCUSSION

The results show that the extraction of diodone from the plasma by the isolated kidney is incomplete, on the average, only 62% being extracted during one renal passage. Also it seems likely, on the whole, that the red blood corpuscles are not concerned in this extraction. The diodone clearance is therefore less than the true plasma flow. Since the assumption is often made that diodone clearance and renal plasma flow are identical in man and in the intact dog it seems important to look for explanations of the incomplete extraction. As Smith [1941] has pointed out, the calculated clearance (and a similar argument can be applied to the extraction ratio) is the over-all clearance, i.e. it is the clearance of plasma which has gone through active as well as possibly inactive parts of the kidney. If there are some nephrons still supplied with blood but from any cause inactive as secreting units, then no diodone will be extracted from the plasma. But if it be supposed that this plasma ultimately mixes with plasma from other units where the extraction has been complete then, obviously, the calculated clearance is less than the total plasma flow according to the proportions of blood flowing through the inactive and the active regions respectively.

On the other hand, all the nephrons in a kidney may be equally active but incapable of effecting a complete extraction of diodone from plasma even at low plasma iodine concentrations. From considerations of the observations which have been made it is possible that this is the condition of the perfused kidney. The progressive decline in the extraction ratio at constant perfusion pressure whatever the direction of spontaneous change in blood flow or alteration in renal plasma load, coupled with the elevation of extraction ratio when the perfusion pressure and blood flow are suddenly reduced, points to this conclusion, as does also the slow rate at which C_D rises to its maximal value after the first addition of diodone to the blood. Evidence is not wanting that the behaviour of the perfused kidney deviates in several respects from that of the normal organ; for example, the polyuria and low chloride content of the urine [Starling & Verney, 1922] and the difference in clearances between inulin and creatinine at high urine/plasma concentration ratios [Shannon & Winton, 1940]. To these, the inability to extract diodone to the same extent as the normal kidney may now be added. There is no evidence to show if this alteration in activity is due to the gradual disappearance of hormones from the circulating blood or to other changes in the defibrinated blood used for perfusion, but it is perhaps noteworthy that complete hypophysectomy reduces the ability of the dog kidney to transfer diodone [White, Heinbecker & Rolf, 1941].

Obviously it is unwise in the light of these findings to assume, particularly when dealing with suspected abnormal functioning of the kidney, that C_D is

equivalent to R.P.F. C_D may be only a relatively small fraction of R.P.F. It is better to relate C_D to the functionally intact renal tissue [Smith, 1941] which may be assessed by measuring the maximal tubular excretion of diodone $[T_mD]$ at high plasma concentrations. It has not been possible to undertake such measurements in the series of experiments reported here.

SUMMARY

1. The mean plasma extraction ratio of diodone by the perfused isolated dog kidney is 0·62 at plasma I levels of 1–2·5 mg./100 ml. This is lower than similar ratios observed in the explanted kidney.

2. The extraction ratio is elevated when the perfusion pressure is lowered and the renal plasma load diminished.

3. It is suggested that the ability of the renal cells to transfer diodone is progressively diminished during the course of an experiment. This may be due to gradual disappearance of hormonal influences.

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PROCEEDINGS

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13 *February* 1943

The physiology of shivering. By R. J. S. McDowall
(*King's College, London*)

If cold saline is put into a plethysmograph containing the skinned limb of a chloralosed cat the muscles undergo irregular contractions reminiscent of shivering. These contractions, which are recorded by the plethysmograph, are abolished by the injection of hot saline into the circulation. They are independent of the nervous system and are exhibited by pieces of muscle removed from the body altogether. It would seem that shivering is not so dependent on the central nervous system as is generally supposed but the reaction is abolished by deep anaesthesia.

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3 April 1943

A simple rocking dialyser. By H. P. GILDING AND K. A. WEBB.
(Department of Physiology, the Medical School, Birmingham)

The figure below is a drawing of the dialyser. The dialysing chamber consists of a glass tube of 550 ml. capacity, diameter 6·5 cm. It is closed by rubber bungs through which the inflow and outflow tubes are passed. The glass tube is mounted on a wooden board and held in place with Terry's clips of an
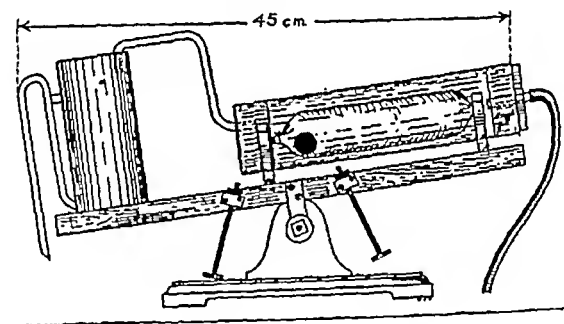
appropriate size. The whole is pivoted on a bearing placed approximately in the centre of its long axis. The inflow tube can be connected either to tap water, or to a large reservoir of distilled water. The outflow tube is bent as shown in the diagram, the water flowing into a metal cylinder of approximately 300 ml. capacity. A metal tube of 6 mm. internal diameter is attached to the bottom of the metal cylinder and bent to make a siphon. A cellophane envelope contains the material to be dialysed, and also a glass bead. The dialysing chamber can be moved in the Terry's clips so that when the metal cylinder is empty, the apparatus tips to the inlet side. As the metal cylinder fills, the whole board rocks over to the outlet side, and the glass bead stirs up the fluid in the cellophane envelope. For accurate adjustment of the angle of tipping required, two threaded metal rods are fastened to either side of the bearing and by altering the screws it is possible to adjust the tilting. In use, the maximum rate of tipping is about once per minute, and the volume of fluid passing through the dialyser at this rate is 300 ml./min.

An ergograph for teaching purposes. By K. A. WEBB.
(*Department of Physiology, the Medical School, Birmingham*)

The figure. which is a scale drawing, shows a simple, easily made, form of ergograph. It is essentially a lever (*A*) fixed to a bearing (*B*) at one end, which presses down a spring (*C*), and a record of the movement of the lever is obtained by a thread attached near the fingergrip of the lever, passing over two pulleys to operate a writing point (*D*). The spring is an Austin 7 valve spring,
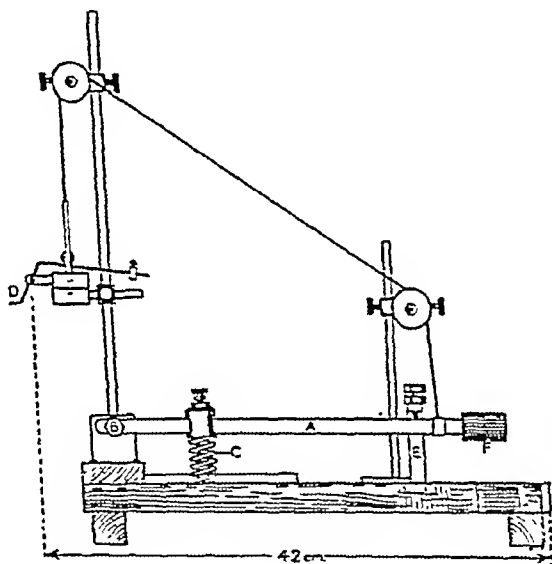
so arranged that the base runs in a guide on the baseboard, and its upper part is attached to a slide which moves along the lever. To stop backlash a rigid arm (*E*) is fastened to the baseboard and bends over the lever, which can be adjusted by turning the thumbscrew, thus taking up the early soft period of the spring. The lever may either be operated by the forefinger placed over the rubber fingergrip (*F*), and for this purpose the baseboard is cut away to accommodate the thumb, and second, third and fourth fingers; or else by placing the fingers beneath the baseboard, the thumb can operate the lever. The spring can be moved along the guides so that the weight required to depress the lever by finger or thumb to its fullest extent varies from 1·5 to 13 kg., and graduated marks on the lever indicate the weight required at a given position of the spring. The frontal writing lever is fastened to a metal block pierced by two holes which slides up and down the guides fixed to the lower block as shown in the diagram.

Effects of 'CTAB' (Cetyltrimethylammonium bromide) on cells in vitro. By F. Jacoby. (*Department of Physiology, the Medical School, Birmingham*)

The use of CTAB as a disinfectant and cleaning agent in surgery has recently been advocated by Barnes [1942] who showed that by its use (1% sol.) dirty dishes, etc. were completely sterilized and skin almost freed from bacteria; but short application to a granulating wound failed to sterilize it, though leucocytes in vitro are, according to a brief reference, rapidly broken up and destroyed.

In the present experiments CTAB (in final conc. from 10^{-3} to 10^{-7}) was applied for 24 hr. (a) to pure populations of hen blood macrophages living in Carrel flasks in 30% serum, and (b) to fibroblastic cell colonies (from chick embryo heart explants) growing in Carrel flasks in a plasma coagulum and fed with embryo extract. After CTAB removal fresh feeding was given, and in case of the fibroblasts this was followed, 24 hr. later, by transplantation. For results see Table I.

TABLE 1

Effects of adding CTAB on

CTAB f.c.	Culture medium	Macrophages	Fibroblasts
10^{-3}	Immediate heavy precipitate; clouding of coagulum	Killed and 'fixed', almost life-like	Killed and 'fixed', many life-like, some rounded off*
10^{-4}	Clouding of feeding fluid and coagulum	Some fatty change precedes death and 'fixation'	Break up, round off, killed and 'fixed'*
10^{-5}	Remains almost clear	After short survival round off, shrink, tend to break up, thus 'fixed'	Growth-zone killed; few cells grow out after transplantation
10^{-6}	Clear	Slight granulation, mitoses proceed	Amount of outgrowth reduced against controls. Recovery after transplantation
10^{-7}	Clear	Normal morphology, mitoses frequent	

* No outgrowth after transplantation.

In the macrophage cultures, where there is no coagulum, CTAB application is a more direct one than in the fibroblast colonies which are (a) protected by a coagulum, and (b) contain sheltered cells in the interior. Hence the survival of some such cells in 10^{-5}, which should not be interpreted as indicating greater resistance. Of special interest is the 'fixing' effect of CTAB which in falling concentrations results in a paradoxical appearance of the treated cells. The obviously deleterious action of CTAB in the higher concentrations seems to be linked up with its protein precipitating and 'fixing' properties; this may also account for its disinfecting action. In transferring the above results to wound conditions great reserve is necessary. Repeated or prolonged application of CTAB (10^{-2} to 10^{-5}) to infected wounds might presumably lead to their sterilization, unless CTAB is rapidly inactivated by wound secretion (e.g. certain phospholipids have been reported to inhibit detergents like CTAB [Baker, Harrison & Miller, 1941]). The damage to living tissue cells, which will probably

affect only very superficial layers, should not weigh too heavily against the advantage gained from the destruction of bacteria.

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The taking of blood for haemoglobin estimation. By J. M. PETERSON, D. H. STRANGEWAYS and R. C. JORDAN. (*The Physiology Institute, and the Anatomy Department, University College, Cardiff*)

An investigation of the variations in haemoglobin content of blood drawn by different procedures from three individuals has been made. The estimations have been carried out by a technique involving the use of a photo-electric absorptiometer whereby replicates of 0·02 c.c. from the same sample of human blood can be analysed with a standard deviation of 0·14 g. Hb/100 c.c. of blood.

The following conclusions have been made:

(1) Replicate samples of finger blood, made temporarily static by a constricting band, or of ear blood from the same individual show the same standard deviation of about 0·4 g. Hb/100 c.c., which is markedly greater than the analytical error shown above.

(2) The mean haemoglobin content of samples of free-flowing blood obtained from the finger without a constricting band does not differ from that of samples taken during constriction although the standard deviation obtained for the former is less than that with constriction by 0·12 g. Hb/100 c.c. blood.

(3) Blood samples squeezed from the finger through needle punctures have the same mean haemoglobin content as free-flowing samples and, again, the standard deviation obtained for the free-flowing samples is less than for those obtained by squeezing by 0·14 g. Hb/100 c.c.

(4) It appears unlikely that brief and complete venous occlusion causes any change in the haemoglobin content of venous blood.

Changes can be brought about, however, by prolonged stasis. Two methods of causing stasis of the circulation of the arm have been employed. By one method a sample of blood is obtained which has been static in the veins for 5 min. and is presumably not affected by change in capillary blood. In the other the capillaries are subjected to a high blood pressure for 3 min. and blood which has been in the capillaries during stasis is allowed to flow into the veins before sampling.

The samples obtained by either method sometimes show differences in haemoglobin content from those obtained with minimum stasis. The first method seems to give a reduction in haemoglobin concentration probably by sedimentation in the vein, and the second usually, but not always, a rise probably due to haemoconcentration in the capillaries.

The authors find no foundation for objections to temporary vascular stasis or squeezing in the taking of blood samples for haemoglobinometry except that the standard deviation may be slightly increased.

PROCEEDINGS

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PHYSIOLOGICAL SOCIETY

17 *July* 1943

A method for the investigation of the action of antiseptics on the growth of epithelial tissue. By J. M. Robson. *From the Department of Pharmacology, University of Edinburgh*

An essential property of any antiseptic to be used in the treatment of lesions of the living body is that it should produce its action on micro-organisms without interfering with the repair processes. With the following method, quantitative data on the effect of various substances on the growth of epithelial tissue *in vivo* can be obtained.

The vaginal epithelium of mice consists of but two layers of flat epithelial cells readily accessible to substances introduced into the vagina. The subcutaneous injection of 0·5 mg. of oestradiol dipropionate produces proliferation
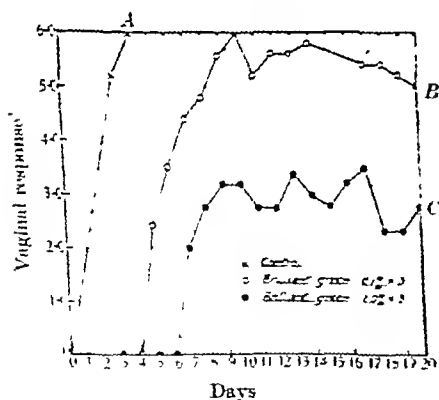
Fig. 1. Showing the effect of brilliant green on the growth of the vaginal epithelium. Each curve represents the average for five mice. Curve *A*: control mice; vagina untreated. Curve *B*: three applications of 0·02 c.c. 0·1 % of brilliant green to the vagina on three consecutive days, the first being given at the time of injection of oestradiol dipropionate (0·5 mg.). Curve *C*: three applications of 0·02 c.c. 1·0 % of brilliant green to the vagina on three consecutive days, the first being given at the time of injection of oestradiol dipropionate (0·5 mg.).

of the vaginal epithelium which is maintained for several weeks, the mice remaining in full oestrus for at least 3 weeks. The condition of growth of the vaginal epithelium can be determined by taking vaginal smears, and estimated in a semi-quantitative manner by a method previously described [Robson, 1938] for evaluating these smears. Following the administration of the oestrogen, the maximum value is reached in a few days and maintained for more than 3 weeks (curve *A* in Fig. 1).

The application to the vagina of substances which interfere with the growth of the epithelium produces an inhibition of the growth response to the standard dose of oestradiol dipropionate given at the same time, and characteristic curves are obtained with various antiseptics. Brilliant green and gentian violet produce marked inhibition of epithelial growth as judged by this method. The effect of acriflavine and proflavine is much smaller, while repeated applications of sulphonamides (sulphanilamide powder, sulphathiazole, 30 % solution of sodium sulphacetamide) do not in any way interfere with the epithelial growth.

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A factor in pancreatin which influences the growth of tissues *in vitro*.

By J. N. DAVIDSON and C. WAYMOUTH. *From the Physiology Department, University of Aberdeen*

Crystalline ribonuclease has been found [Davidson & Waymouth, 1943] to have no influence on the growth-promoting properties of embryo extract towards fresh explants of the 9-day chick embryo heart cultivated *in vitro* according to the roller-tube technique of Willmer [1942]. On the other hand, crude ribonuclease preparations prepared from pancreatin [Dubos & Thompson, 1938] have been found to contain a factor which influences markedly the growth of the cultures. Cultures grown in a mixture of embryo extract and the factor have a smaller area, a more compact and dense appearance and a much higher nucleoprotein content than control cultures grown in embryo extract alone. The cells of the treated cultures are well nourished and polyhedral in appearance and show more mitotic figures than the more elongated cells of the control cultures.

The material which produces this effect can be purified by extraction from aqueous solution with phenol. It is stable to heat in slightly acid solution, but its activity is destroyed after heating at 100° for 30 min. in faintly alkaline solution. It contains 13·6 % N and 2·0 % P. Of the total N 13·0 % is present in the form of amino N and this figure rises to 58·7 % after acid hydrolysis. Tests for arginine and tyrosine are positive.

The characteristic effect produced by this material has not been given by any other substance so far tested, including phosphopeptone prepared from casein, insulin and the ribonucleic acid of the pancreas.

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The effect of progesterone on the guinea-pig uterus. By J. ADLER and G. H. BELL. *From the Institute of Physiology, University of Glasgow*

't has been shown [Bell, 1941] that the threshold dose of oxytocin required to produce a contraction of the uterus of the guinea-pig *in vivo* decreases as, pregnancy advances. The period of low reactivity coincides with the period of activity of the corpus luteum. Bell & Robson [1936] found, however, that progestin had no effect on the reactivity of the non-pregnant guinea-pig uterus *in vivo*. They used either crude luteal extracts or small amounts of progesterone which had then just become available. As this negative finding might have been due simply to inadequate dosage of the hormone, these experiments have been repeated using much larger doses—up to a total of 30 mg. progesterone over 3 days. The results are given in the table. In spite of the great variability

Threshold dose of oxytocin o.u. ...	Up to 0·01	Over 0·01 to 0·1	Over 0·1
No. of animals treated with oestrin or with large follicles in ovaries	9	3	0
No. of animals treated with progesterone or with corpora lutea in ovaries	3	7	2

of the results there is a statistically significant tendency for the progesterone-treated uteri to be less reactive. The overlap of these findings with those obtained in the pregnant uterus renders it unnecessary to invoke the presence of a new hormone in early pregnancy to explain the unreactive phase. Further, the amplitude of the spontaneous contractions of the progesterone-treated uteri varied much more from animal to animal than did the amplitude in the oestrin-treated uteri. These findings on reactivity and activity suggest that the progesterone-treated uterus is more easily influenced by environmental conditions, e.g. state of growth or tension, than is the oestrin-treated uterus.

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Haemalum-aurantia-aniline blue. A general-purpose staining method. By A. MARSHALL and O. A. TROWELL. *From the Physiology Department, University of Edinburgh*

The Mallory, Azan, Masson and van Gieson methods achieve a selective staining of collagen fibres, but the staining of the other tissue elements is somewhat diffuse. The method to be described provides the selective collagen staining of the Azan method together with a precise and colour-differentiated staining of nuclei, muscle cytoplasm, epithelial cytoplasm and red blood cells.

The method, which is applicable to paraffin sections of any organ after any of the ordinary fixatives, is as follows:

(1) Haemalum (Mayer's), 15 min., followed by tap water.

(2) Aurantia (ammonium hexanitro-diphenylamine) (B.D.H.), 0·5 % solution in 70 % alcohol, 5 min. or longer. Wash.

(3) Phosphotungstic acid 5 %, ½–1 hr. Wash.

(4) Aniline blue, 0·25 % solution of water-soluble aniline blue (Gurr) in 0·2% acetic acid, ½ hr. Wash.

(5) Blot off as much water as possible.

(6) Dehydrate in terpineol, 2 min. Wipe off surplus.

(7) Xylol, a few min. only. Mount.

Alcohol dehydration is avoided because in the case of some organs the phosphotungstic acid renders the aurantia staining alcohol-soluble. Terpineol is the only dehydrating agent discovered which has no effect on the aurantia. A jar of terpineol will last a long time if a few lumps of anhydrous calcium chloride are added to it.

Results. Nuclei dark brown, muscle cytoplasm golden yellow, epithelial cytoplasm pale purplish brown, collagen dark blue, elastic laminae (e.g. arteries) bright yellow, red blood cells bright orange.

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2 *October* 1943

Measurement of renal blood flow. By C. A. KEELE and D. SLOME.
*(Departments of Physiology and Pharmacology, Middlesex Hospital Medical
School, London)*

Two methods used in anaesthetized cats are described.

(1) *Plethysmograph and venous occlusion.* The kidney is enclosed in a plethysmograph and connected to a volume recorder. The method of Brodie &
Russell [1905] is used, the renal vein being temporarily occluded by an electromagnetically operated compressor. The rate of increase of volume recorded
is a measure of renal blood flow which is finally expressed as c.c./100 g. of
kidney/min.
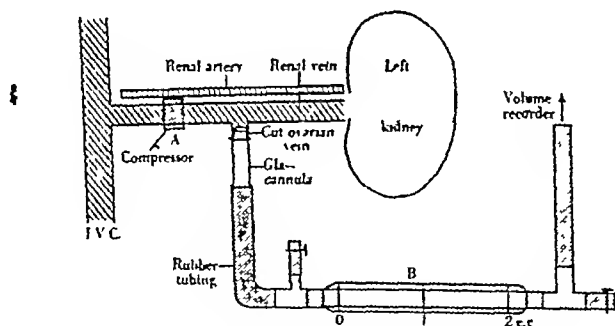
Fig. 1. Diagram of venous shunt method.

(2) *Venous shunt.* In some cats, especially pregnant ones, the left ovarian
vein can be cannulated close to its site of junction with the left renal vein (see
Fig. 1). Complete obstruction of the renal vein at *A* causes the blood from the
kidney to be shunted via the cannula and the rate of flow can be measured
directly. The animal is heparinized, a glass cannula inserted, and then the
cannula, rubber tubing and graduated glass tube *B* are filled with blood to
point 0 by temporary obstruction of the renal vein. Glass tube *B* is then
placed horizontally at a height which just balances the pressure in the renal
vein so that the blood level remains at point 0. Complete obstruction at *A*
causes blood to flow along tube *B*, and the time taken for 1 or 2 c.c. can be

measured with a stop watch, or the distal end of tube B can be connected by air transmission to a volume recorder writing on a moving smoked drum. The latter method is preferable as the slope of the recorded line is a continuous measure of rate of flow. The blood level is restored to 0 by temporarily raising tube B. The advantages of this method are that renal blood flow is measured directly with removal of only a few c.c. of blood from the animal, that no manipulation of the kidney is required, and that venous congestion is avoided.

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Diuretic action in man of 'old' post-pituitary extract. By J. MARKS.
(Department of Physiology, Middlesex Hospital Medical School, London)

A commercial extract of posterior pituitary prepared in 1931 gave unusual diuretic responses when injected 11 years later in man. It also produced flushing of the face, pyrexia and a feeling of cold and shivering lasting some hours. The specimen was sterile though the possibility of previous bacterial contamination could not be excluded.
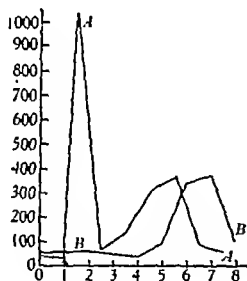
Fig. 1.   Fig. 2.

Fig. 1. Ordinate: volume of urine in c.c./hr.
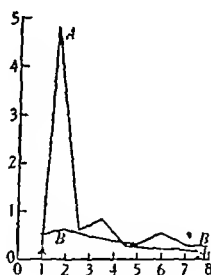
Fig. 2. Ordinate: total chloride excretion in urine in g./hr.

In both figures, abscissa: time in hours. At arrow in each figure inject 5 units of 1931 pituitrin (curves A) or 1942 pituitrin (curves B) intramuscularly and 1 l. of water drunk.

(1) In a subject on a standardized diet and water intake, 5 units of the 'old' extract increased the flow of urine during the next 3 hr. above the maximum level of a 24-day control period; there was a significant increase in urinary chloride concentration and in total output. Fresh pituitary extracts decreased the urinary flow below the control minimum for 5 hr. (without subsequent diuresis) and raised total Cl excretion less markedly. Typhoid vaccine injections which produced the same general reactions as the 'old' extract decreased the urinary flow.

(2) Given to subjects after ingestion of 1 or 2 l. of water, the 'old' extract produced an initial diuresis (generally greater with bigger doses) reaching its peak after ½ hr. with a flow at rates up to 1000 c.c./hr. (Fig. 1, *A*). There was only a small fall in urinary specific gravity or in Cl concentration and there was consequently a large rise in total Cl output (Fig. 2. *A*). The increase in urea excretion was less evident. The urinary changes were unrelated to the circulatory alterations. After a variable interval the secondary diuresis set in which was identical in character with the single delayed diuresis following injection of fresh preparations.

The peculiar pharmacological properties of the 'old' extract were not due to simple ageing of the sample as other specimens of the same date and serial number produced normal uncomplicated antidiuretic effects. Exposure of fresh preparations to air for 75–95 days did not modify their activity.

Effects of histamine and insulin on gastric secretion, with gastroscopic observation of the secreting stomach. By A. MORTON GILL.

(Department of Physiology, Middlesex Hospital Medical School, London)

Histamine acts directly on the gastric mucosa; insulin by producing hypoglycaemia sets up a nervous secretion reflexly via the vagi.

Technique. All observations were carried out on fasting subjects using continuous aspiration, after preliminary removal of the resting juice. Soluble insulin (7 units) is given intravenously at 40 min. and histamine 0·5 mg. subcutaneously 20 min. later; since hypoglycaemia develops in 30–40 min. after intravenous insulin and the histamine reaction takes 10–20 min. to develop, this timing ensures a summation of nervous and chemical secretion. The total amount collected each 10 min. is measured and titrated and a graph, showing volume and acid concentration, plotted. Less than 7 units of insulin produces insufficient stimulation, with more than 9 units hypoglycaemia may be alarming; 7 units is optimal. The volume of juice secreted per min. for the 60 min. following the histamine injection has also been estimated. Each experiment lasts 160 min. to ensure that the effects of both insulin and histamine have ceased.

Material and results. One hundred and sixty-one individuals were examined including thirteen normal controls, the remainder having gastroduodenal ulceration or inflammation; in all a fractional gruel test meal was also performed, and gastroscopic examination of the resting stomach. The average normal response to the double stimulation was a maximum free HCl concentration of 100–120 cc. $N/10$ HCl/100 c.c. of gastric juice, and a secretion rate of 2·5–3·5 c.c./min. The degree of response followed closely the type of mucosa, being greatest (180–200 c.c. $N/10$ HCl % and secretion of 6–7 c.c./min.) in individuals whose mucosa was hypertrophic, minimal in those with mucosal atrophy following long-standing chronic gastritis. Of twenty-five individuals showing complete achlorhydria after gruel, only those with extensive diffuse mucosal atrophy failed to secrete HCl after histamine ÷ insulin; of these twenty-five, four were otherwise normal and their histamine ÷ insulin gastric

response (H.I.G.R.) was likewise normal. Repeated H.I.G.R. on the same individual have shown a reasonable degree of constancy; the response after histamine + insulin is always greater than after either stimulus alone. Gastroscopic observation of the normal stomach after histamine + insulin shows no change in the character or colour of the mucosa, which exudes a clear fluid, forming rivulets between the folds and trickling down to form a pool in the most dependent portion of the stomach. A similar response was obtained after the ingestion of 2 fluid oz. of concentrated meat extract, secretion proceeding without visible alteration of the mucosa. However, in view of the failure to obtain vasodilatation and the fact that this follows vagus stimulation in animals, all the above gastroscopic observations were repeated, gastroscopy being performed from 1 to 2 hr. after histamine and insulin, after the ingestion of concentrated meat extract and after the ingestion of a light lunch consisting of fish, potato and rice pudding; in every case a diffuse hyperaemia of the mucosa was found. It would therefore appear that, in man, secretion begins before vasodilatation becomes visible.

Magnesium-calcium antagonism in blood clotting. By G. D. GREVILLE and H. LEHMANN. (*Runwell Hospital, nr. Wickford, Essex*)

The addition to blood of neutral salts in high concentration (final molarity 0·2 and higher) prevents clotting, which can then be induced by dilution. We have found that $MgCl_2$ is an anti-coagulant at lower concentration than the above. Provided that the concentration is not too high, its effect can be overcome by the addition of $CaCl_2$ as well as by dilution.

When human plasma, obtained without anti-coagulant, was mixed with 1/10 vol. of 0·5 M $MgCl_2$, giving a final concentration of 0·045 M, and left at room temperature, no clot appeared in 10 days. 0·05 M $MgCl_2$ was also effective with whole blood. When this concentration was obtained by adding 1 vol. of $M/7$ $MgCl_2$ to 2 vol. of blood, the cells remained undamaged, as judged from stained films and hanging drops. These findings may therefore be of practical value.

The prevention of clotting by Mg^{++} is due to a competitive inhibition of Ca^{++} activation. We have established this in various ways. The simplest experiment shows that $CaCl_2$ added in a concentration which does not accelerate the normal clotting rate can overcome a complete inhibition by $MgCl_2$.

Each tube contained 1 ml. human plasma, obtained by centrifuging blood in cooled paraffined tubes, with additions as below. Temp. 22° C.:

	1	2	3	4
Water (ml.)	0·2	0·1	0·1	—
0·03 M $CaCl_2$ (ml.)	—	0·1	—	0·1
0·4 M $MgCl_2$ (ml.)	—	—	0·1	0·1
Clotting time	13 min.	13 min.	∞ (>3 days)	135 min.

These ion effects resemble those in the adenosine triphosphatase of myosin. Here again Ca^{++} activates [Needham, 1942]. and Mg^{++} inhibits by competing with the Ca^{++} [Greville & Lehmann, 1943]. Further, supra-optimal concentrations of $CaCl_2$ inhibit both myosin activity [Greville & Lehmann, 1943] and blood clotting [Horne, 1896]. Presumably both these processes involve at one stage the formation of a complex between Ca^{++} and two organic molecules: adenosine triphosphate and myosin in the one system, thrombokinase and prothrombin in the other.

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The definition of the National Physical Laboratory Haldane haemoglobin standard. By C. G. DOUGLAS. E. M. JOPE, H. M. JOPE, R. G. MACFARLANE and J. R. P. O'BRIEN

The following estimations were made on forty-nine blood samples from normal adults:

Oxygen capacity, by the van Slyke constant volume apparatus, checked by carbon monoxide capacity. In eleven cases readings were also made on the Haldane gas analysis apparatus, which gave a mean figure 0·35 c.c. % lower than the corresponding estimations on the van Slyke apparatus.

Haemoglobin iron, by the $TiCl_3$ method, against standard iron wire.

Haemoglobin, by Haldane's colorimetric method, against the National Physical Laboratory standard.

Red cell count and haematocrit using standardized apparatus.

Ultra-violet and visible absorption spectra by means of a Hilger E3 medium quartz spectrograph, and Spekker photometer (ribbon filament lamp), the plates being matched on a photoelectric micro-photometer. The results in the visible range agree closely with estimations made at the National Physical Laboratory.

From these results it is calculated that blood equivalent to 100% of the N.P.L. standard has the following mean values: 19·88 c.c. O_2%; 49·83 mg. Fe%; 5·17 million R.B.C./c.mm.; 44·3% P.C.V.; HbO_2, $E_{1cm.}^{1:1000}$ ($\lambda = 414$ mμ) 1·200.

Six samples of blood, in which the haemoglobin was estimated at the N.P.L. by direct comparison with the standard colour tube by means of their visual colorimeter, gave the following values equivalent to 100% Hb: 19·67 c.c. O_2%; 48·20 mg. Fe%; HbO_2, $E_{1cm.}^{1:1000}$ ($\lambda = 414$ mμ) 1·180; $HbCO$, $E_{1cm.}^{1:100}$ ($\lambda = 540$ mμ) 1·271.

The difference between these two sets of results is presumably due to the slightly lower readings of haemoglobin estimated by Haldane's dilution method, as compared with those obtained on the N.P.L. colorimeter.

Haemolysins in urine. By Eric Ponder.
(*Nassau Hospital, Mineola, N.Y.*)

Haemolytic substances in urine were first described by McKee [1915], who thought that their appearance might have some pathological significance, and were found to occur in over 90 % of normal human urines by Ponder [1921 a,b]. Abels [1934] showed that the lytic substance is heat stable, soluble in ethyl alcohol but not in most organic solvents, adsorbable on activated charcoal, inhibited by plasma proteins, cholesterol and lecithin, and inactivated at pH 8·0–9·0. On the basis of these and other properties he tentatively identified it as the lysin of *B. coli*.

I have been able to isolate a haemolytic organism from every one of a large number of actively haemolytic urines obtained from supposedly normal individuals. The methods used for determining the lytic activity were the same as those previously used by me and by Abels, and a urine was considered actively lytic if 0·2 ml. produced complete lysis in the usual test system in 3 hr. at 37° C. These urines were invariably acid. Each was plated out on blood agar, and any haemolytic colonies which appeared were transferred to appropriate media so as to give pure cultures. The organisms, in pure culture, were then suspended in saline and shown to be lytic for the test system. Sometimes no haemolytic colonies appeared on the blood agar, in which case each of the prevailing types of colony were grown in pure culture and tested in suspension for haemolytic activity. One has to be cautious in labelling an organism as non-haemolytic, because many bacteria (e.g. *B. coli*) are haemolytic only at certain stages of their growth.

In about four-fifths of the cases the lytic organism was identified as *B. coli communis*. In the remainder, it was *Streptococcus haemolyticus* or *B. proteus*, except in a few cases in which the bacterium was not identified. The frequency with which bacteria, and *B. coli* in particular, can be isolated from apparently normal urines has been pointed out by Marple [1941]. Haemolytic strains may occur even in catheter specimens of urine, and I have not yet found a urine to be haemolytic when no growth could be obtained in culture.

The observation of Abels & Rhoads [1938], that the urine of persons suffering from aplastic anaemia is characteristically non-lytic, but that it becomes lytic on boiling with acid, requires further elucidation.

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1

A protein of low density prepared from human serum. By G. S. Adair and Muriel E. Adair. (*Physiological Laboratory, University of Cambridge*)

Roche, Dorier & Samuel [1936] reported that in order completely to precipitate globulin from human serum, 60% saturation with ammonium sulphate is required.

Human serum was precipitated with 33% saturation of ammonium sulphate to remove euglobulin and then with 50% saturation to remove pseudo-globulin. We found that if the saturation of ammonium sulphate were then raised to 60% a small quantity of precipitate formed and on standing rose to the surface of the fluid. the density of which was 1·17. Previous investigations [Adair & Adair, 1936] have shown that densities exceeding 1·23 were required for the flotation of euglobulin and of serum albumin. The light fraction was soluble in media of low densities. but by an indirect method which will be described in a later paper it was found that the density might be as low as 1·10.

A fraction purified by reprecipitation appeared to be almost homogeneous when investigated in the Tiselius cataphoresis apparatus, after dialysis against $M/15$ phosphate buffer pH 6·8. The nitrogen content of this preparation was 6·44%; organic phosphorus was 0·67%. Lipoid analyses gave 8·5% of phospholipoid, 16·5% of cholesterol and 20·4% of fatty acids. We are greatly indebted to Dr Popják for the lipoid analyses. The lipoid content is thus considerably higher than that reported for β globulin by Blix, Tiselius & Svensson [1941].

The molecular weight of our preparation, estimated from the osmotic pressure, was 370,000. The membrane potentials were approximately equal to those of serum albumin, at the same concentrations of nitrogen.

Serological tests of the material were carried out against an antiserum prepared by injection of crystalline human serum albumin into a rabbit and against an antiserum prepared by injection of total human globulin. The globulin was obtained by 50% saturation of human serum with ammonium sulphate and had been reprecipitated twice.

Both antisera reacted rapidly with their homologous antigens. With the protein of low density. only trace reactions were obtained after long standing.

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The effect of fasting on the protein content of the liver. By H. W. Kosterlitz and Isobel D. Cramb. (*Department of Physiology, Marischal College, Aberdeen*)

Addis, Poo and Lew [1936] found that rats fasted for 2 days lost 20% of the protein content of their livers while the loss of protein in the other organs amounted to only 4%.

In the present investigation, an attempt was made to correlate the loss of liver protein caused by fasting with changes in the phospholipin and nucleoprotein contents.

Male hooded rats of 400 to 450 g. body weight, fed on the stock diet or fasted for 24 or 48 hr., were killed by decapitation. The ground livers were first extracted with trichloroacetic acid; the residue was extracted twice with cold alcohol, twice with hot alcohol-ether mixture (3 : 1) and once with warm ether. Phospholipin-P was determined in this extract. Protein-N and nucleoprotein-P were estimated in the residue.

The results are given in Table 1.

TABLE 1

	Relative liver weight g. liver/100 g. original body weight (before fasting)	Liver phospholipin-P mg./100 g. original body weight	Liver nucleoprotein-P mg./100 g. original body weight	Liver protein-N mg./100 g. original body weight
Fed rats (6)	3.46 ± 0.13	4.79 ± 0.13	3.79 ± 0.12	94.9 ± 2.2
Rats fasted for 24 hr. (4)	2.43 ± 0.09	3.81 ± 0.20	3.31 ± 0.10	77.3 ± 3.3
Loss (%)	-29.8	-20.5	-12.7	-18.6
Rats fasted for 48 hr. (6)	2.32 ± 0.05	3.55 ± 0.13	3.11 ± 0.09	72.2 ± 2.2
Loss (%)	-33	-25.9	-17.9	-23.9

	$\dfrac{\text{Protein-N}}{\text{Phospholipin-P}}$	$\dfrac{\text{Protein-N}}{\text{Nucleoprotein-P}}$	Nuclei in 10^{-3} c.mm. embedded liver	Nuclei in 10^{-3} c.mm. embedded liver × relative liver weight
Fed rats (6)	19.8 ± 0.4	25.0 ± 0.2	170 ± 8	588
Rats fasted for 24 hr. (4)	20.3 ± 0.5	23.4 ± 0.4	248 ± 15	602
Loss (%)	—	—	—	
Rats fasted for 48 hr. (6)	20.3 ± 0.2	23.2 ± 0.4	259 ± 19	600
Loss (%)	—	—	—	

No differentiation was made between cytoplasmic and nuclear nucleoproteins which may be lost at different rates. The decrease in liver weight is not caused by a decrease in the number of liver cells but by a decrease in the volume of each individual cell.

In another series of experiments on 12 female rats ranging in weight from 127 to 250 g. and kept under varying nutritional conditions, the ratio protein-N/phospholipin-P was 20.6 ± 0.2 while the ratio protein-N/nucleoprotein-P varied from 18.4 to 24.5. This remarkable constancy of the former ratio suggests that, although the 'stored' protein is readily given up by a 24 hr. fast, together with the phospholipins it probably forms a structural part of the cytoplasm.

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The effect of trauma on the release of histamine from blood cells
in vitro. By G. UNGAR. (*Department of Human Anatomy, University of Oxford*)

Gotzl & Dragstedt [1942] have shown that appreciable amounts of histamine are released when rabbit blood is mixed with peptone. This reaction provides a convenient method for the study of certain problems connected with systemic changes resulting from trauma.

Blood was collected by cardiac puncture and coagulation was prevented by heparin or 'Liquoide'; 0·5 c.c. of each sample was mixed with 1 c.c. of saline (*A*) and another 0·5 c.c. with 1 c.c. of a 1·3% peptone solution (*B*). After centrifuging for 10 min., the supernatant fluid was collected and diluted to 5 c.c. Histamine was estimated by the usual technique, using the guinea-pig ileum as test object. Results are given in μg. of histamine dihydrochloride per c.c. of blood. The amount of histamine released is given by the difference between the two samples, taking into account the direct action of peptone (*P*) on the intestine ($H = B - A - P$).

Table 1 confirms that histamine is released *in vitro* from the blood of normal guinea-pigs, and shows that the amount is reduced (*a*) in the refractory phase following the injection of peptone, and (*b*) after trauma administered under ether anaesthesia [Ungar, 1943]. Restoration of the normal rate of release runs parallel with healing. Haemorrhage without tissue injury has no effect on histamine release.

TABLE 1

	$\mu g./c.c.$	$\pm$ S.E.	No. of animals		$\mu g./c.c.$	$\pm$ S.E.	No. of animals
Control	0·19	0·02	39	Trauma (*cont.*)			
Peptone				0·95 kg.m.			
500 mg./kg.				9–10 days after	0·11	0·03	15
2½ hours after	0·04	0·02	8	19 days after	0·1	0·025	10
24 hours after	0·04	0·03	7	23–24 days after	0·22	0·03	9
100 mg./kg.				0·42 kg.m.			
1 hour after	0·06	0·025	8	3 days after	0·075	0·03	8
20 hours after	0·11	0·02	8	8 days after	0·11	0·03	7
2 days after	0·18	0·04	8	11 days after	0·27	0·08	6
50 mg./kg.				0·21 kg.m.			
2 hours after	0·12	0·05	8	5 hours after	0·05	0·02	8
10 mg./kg.				4 days after	0·11	0·015	7
2 hours after	0·24	0·02	8	6 days after	0·17	0·01	7
24 hours after	0·17	0·05	8	8 days after	0·21	0·05	7
Trauma				0·055 kg.m.			
0·95 kg.m.				5 hours after	0·25	0·03	8
1 hour after	0·22	0·045	8	2½ hours after	0·27	0·025	8
3 hours after	0·225	0·03	8				
4 hours after	0·05	0·02	15	Haemorrhage			
2 days after	0·04	0·02	8	1% body weight			
5 days after	0·06	0·03	8	4 hours after	0·23	0·01	7
				24 hours after	0·23	0·015	8

These facts can be related to the reduction in post-traumatic mortality as a result of previous injury (Noble, 1943; Ungar, 1943).

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The higher centres and the blood-sugar curve. By C. REID.
(*Physiology Department, London Hospital Medical College*)

In the following experiments bearing on the relation of the higher centres to the behaviour of the blood-sugar curve, a mature bitch, weight 12·5 kg., trained to stay quietly on a Pavlov stand, was used throughout. The animal was deprived of food for 24 hr. before the observations were made. An initial sample of blood was taken for the sugar determination and followed by glucose by stomach tube (2·5 g./kg. body weight in 400 ml. water) or into a vein (1 g./kg. body weight). Blood samples were taken at hourly intervals afterwards for 5 hr. Control days without sugar were also run.

These observations alternated with others in which the animal was allowed every ¼ hr. or so to sniff and to taste minimal quantities of finely minced horse flesh. In this way it was hoped to stimulate the islets of Langerhans via psychic mechanisms through their vagus supply.

An experiment was done not oftener than once every week for a period of 12 months.

Illustrative results in one period of 4 weeks:

Type of exp.	Blood sugar mg. %						
	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	
Sugar by stomach tube	82	117	112	101	92	80	
Control	77	80	76	75	77	74	
Sugar by stomach tube	78	106	64	60	58	55	Meat sniffing
Control	80	74	68	66	64	60	and tasting

Statement of results

1. The blood-sugar curves were consistent for any one type of experiment in the series.

2. The sniffing and tasting of meat after giving sugar were followed by a more rapid lowering of the blood sugar than in the corresponding experiments without the exhibition of meat. In addition, sniffing and tasting meat without sugar caused a fall in blood sugar.

3. These results suggest that the output of insulin is increased after activation of the higher centres through the olfactory and gustatory mechanisms.

A physiological factor in food metabolism. By I. HARRIS and
C. E. VERNON (introduced by H. E. ROAF)

In their first paper [1942*a*] McCance and co-worker found a negative Ca balance under brown, after a period of white bread intake; in their second [1942*b*] a decreased absorption. They blamed phytic acid for this unwarrantably. The ions and molecules of the body remain constant within narrow limits.

Absorption of Ca depends also on availability of other substances which compete with each other for the limited space in tissues and blood. Phosphorus in particular stands in reciprocal relation to Ca. Phosphorus and other substances are present in much larger quantities in brown bread. Apparently part of Ca in the body is replaced by phosphorus and some excreted into the bowels, giving rise to a negative balance. We carried out the following experiment: A subject was kept on a constant Ca intake and a constant diet for one period. In a second period under the same Ca intake and diet phosphorus and other substances were added. First period of Ca output 78% of intake. First three days of 2nd period, 160%; ultimately Ca 133%. In the dephytinized bread there has been so much interference that many new factors come into play, and the experiments, therefore, prove nothing.

	White bread period 14 days	National wheatmeal bread without added Ca					
		1st week	2nd week	3rd week	4th week	Last 3 days	
U { Vol. in c.c.	835	1179	1327	1607	1598	1735	Figures are daily averages
{ Ca	32·0	31·5	35·0	37·3	34·4	42·6	and Ca is % output in
Ca in $U+F$	89·5	123·7	155·6	139·5	118·5	114·3	relation to intake. Experiment continues.

The balance experiments of McCance were not carried out long enough. It is clear from our experiments that urinary volume and urinary Ca are raised—a fact incompatible with a mere decreased absorption. Large numbers of people have eaten brown bread all their lives without showing Ca deficiency, which is incompatible with a permanent negative balance.

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The haemoglobin equivalent of the N.P.L. Haldane standard. By E. J. KING, M. GILCHRIST and A. MATHESON. (*The British Postgraduate Medical School, London*)

Substitution of the Clegg and King (1942) alkaline haematin method for Hb (100% Hb = 15·6 g. Hb or 20·9 ml. O_2) for the classical Haldane procedure (100% Hb = 13·8 g. Hb or 18·5 ml. O_2) in routine blood analyses resulted in a much smaller fall in average percentage figures than was expected. This prompted an investigation of the strength of the Haldane standard colour tubes.

Haldane Hb estimations on a large number of bloods were compared with accurate Fe analyses and photometric haematin estimations. Our Haldane colour standards and dilution tubes were standardized by the National

Matthews [1933] has described a similar increase in sensitivity in the stretch endings of striated muscle after giving chloroform. The action on stretch receptors may be a common feature of all volatile anaesthetics, while the occurrence of shallow breathing probably depends on the extent to which other mechanisms are affected simultaneously.

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The relationship of phospholipin to the absorption of unhydrolysed fat from the intestine. By J. J. ELKES* and A. C. FRAZER.* (*Pharmacology Department, The Medical School, University of Birmingham*)

Frazer & Stewart [1939] showed that protein formed a protective layer around a soap-stabilized oil globule. With Elkes & Schulman [1943] they have recently demonstrated that such emulsions flocculate when mixed with protein at a suitable pH, due to the formation of a protein monolayer at the interface. This flocculation can be prevented by the inclusion of phospholipin in the interfacial film.

Macfarlane & Knight [1941] showed that type A *Cl. Welchii* toxin contained a lecithinase which broke the substrate into phosphocholine and a diglyceride. This enzyme caused creaming in a lecithin stabilized emulsion.

Using these two methods, the emulsion formed in the lumen of the intestine and that found in the blood at the height of fat absorption has been studied.

If rats are fed with olive oil and killed 3 hr. later, emulsified fat can be obtained from the intestine and from the blood, and a sample of rat plasma can be prepared. If the intestinal emulsion was tested with rat plasma, flocculation immediately occurred, but with lecithinase no change was seen in 12 hr. A soap-stabilized emulsion introduced into the intestine gave exactly similar results. The chylomicrons on the other hand were separate discrete particles which showed no sign of flocculation. Lecithinase caused complete breaking of this blood-fat emulsion.

It is suggested that the interfacial film of the emulsion in the lumen of the intestine contains no phospholipin—it is·probably an acid-soap film. The chylomicron, however, has phospholipin at the interface as an essential part of the stabilizing film. Frazer [1938] has shown that unhydrolysed fat passes in a finely divided state through the intestinal cell to the lacteals and thence into the systemic blood. Phospholipin appears to be added to the fat globule

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ir transit. From the work of Sinclair [1936] it seems likely that this incorporation of phospholipin into the interfacial film may occur in the intestinal cell.

We wish to thank Miss Macfarlane and Dr Knight for providing us with the lecithinase used in these experiments.

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Oxygen in the blood emerging from the brains of post-mature foetal rabbits. By JOSEPH BARCROFT (*Cambridge*) and I. MAUREEN YOUNG (*Bedford College*)

(1) Barcroft, Flexner, Herkel, McCarthy & McClurkin [1935] showed that in normal pregnant rabbits, the blood emerging from the uterine vein contained progressively less oxygen till at term, when it was reduced to about 30% saturation; this raised the question of how post-mature foetuses, which may be upwards of double the weight of normally born ones, fare for oxygen.

(2) Snyder & Rosenfeld [1937] observed that post-mature foetuses in utero make active respiratory movements, which are abolished by the administration of nitrogen to the mother, from which they, the authors, argued that the movements are not caused by oxygen lack.

Observations were made to throw light on both the above points.

Blood from venous sinuses of foetal brain (*average figures*)

| | Rabbits injected with prolan on 25th day | | | | Control rabbits | | | |
| | | Oxygen | | | | | Oxygen | | |
Foetal age days	Foetal weight g.	Capacity c.c. per 100 of blood*	Content c.c. per 100 of blood	Saturation %†	Foetal weight g.	Capacity c.c. per 100 of blood	Content c.c. per 100 of blood	Saturation %†
		Haldane, standardized by Van Slyke	Van Slyke			Haldane, standardized by Van Slyke	Van Slyke	
27	—	—	—	—	26	12·5	6·3	53
28	33	11·1	6·8	62	—	—	—	—
29	—	—	—	—	46	13·2	5·6	44
30	45	12·7	7·1	62	—	—	—	—
31	—	—	—	—	—	12·1	5·5	43
32	60	15·4	6·9	47	42	17·4	9·0	53
33	59	16·7	5·1	30	—	—	—	—
34	71	16·8	3·3	23	—	—	—	—
35	74	14·0	2·0	15	—	—	—	—
36	79	Dead or moribund			—	—	—	—

* From jugular vein.　　　† Calculation from capacity of blood used for content determination.

To J. Hammond, Jnr., our thanks are due for prolongation of the gestation periods by injection of 100 units of human pregnancy urine extract intravenously and 9 mg. progesterone in oil subcutaneously. To judge of the oxygen atmosphere to which the brains of the foetuses were subjected we collected samples from the venous sinuses by intracranial puncture.

During the period of post-maturity the oxygen saturation of the blood from the brain deteriorates progressively, the oxygen capacity rises up to the 34th day. The foetuses therefore pass through a period of moderate anoxaemia (which may well contribute to respiratory activity) to reach one of profound anoxaemia and ultimate death. The placental mechanism for the supply of oxygen appears unable to keep up with the foetal growth.

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